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It is characteristic of Science and Progress that they continually open new fields to our visions.—PASTEUR.

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ANTIGENIC RELATIONSHIPS OF BACT. TYPHOSUM

II. COMPLEMENT-FIXATION[1]

ELLIOTT STIRLING ROBINSON

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Received for publication February 1, 1923

In a previous communication (Robinson, 1923) are given the results of an attempt to classify or "group" strains of *Bact. ytphosum* on the basis of their agglutinability by several monovalent sera. These experiments demonstrated anew that such strains show marked variation in agglutinability, the variations extending even to cultures derived from the same ancestral strain. Inasmuch as the differences found might conceivably be due to the inherent weakness of the agglutination method, another serological procedure, complement-fixation, has been employed in a study of the same series of typhoid strains.

Complement-fixation as a method for the identification of bacteria possesses one virtue that is lacking in agglutination—namely, the union or non-union of antigen and antibody is evidenced by the reaction of an antigen and antibody of another system, not by an effect on the bacteria under examination themselves. Thus, in the complement-fixation reaction, the indicator of a positive or negative result is the same in all tests and is not subject to variation in a given experiment as is the case in agglutination phenomena. Agglutinin-absorption to some extent enjoys the same advantage as complement-fixation, but it is subject to disturbing factors which make complement-fixation appear the method of choice for the study of this problem.

[1] Dissertation presented to the Faculty of the Graduate School of Yale University in candidacy for the degree of Doctor of Philosophy.

The utility of complement-fixation for the identification of *Bact. typhosum* has been recognized since the method was described by Bordet and Gengou (1901) inasmuch as this organism was one of those employed in their first work. Following them, Ballner and Reibmayr (1908) found the method of value in the differentiation of typhoid and paratyphoid bacilli. Dean (1910), some years later, attempted to detect *Bact. typhosum* in feces by washing off plates inoculated with the fecal material and using these washings as antigens after repeated freezing and thawing. Despite the fact that these antigens contained many more colon bacilli than anything else, his procedure was quite as successful as the ordinary cultural methods in detecting the presence of *Bact. typhosum*.

So far as can be ascertained, the only attempt to differentiate known typhoid strains from each other by complement-fixation tests is that of Hooker (1916–1917). After testing 53 strains with 12 sera, he claimed that races of typhoid bacilli could be distributed into three groups. Disregarding for the moment the interpretation of the results, the fact remains that Hooker was satisfied that complement-fixation is a valuable method for the allocation of strains of *Bact. typhosum*. Various other writers have used or recommended the use of complement-fixation tests for the diagnosis of typhoid infection, but this phase of the subject is not pertinent to the present investigation.

PREPARATION OF REAGENTS

The antigens used were bacterial suspensions prepared from the 48 hour growth of the organisms on extract agar slants. All the strains were grown on slants prepared from a single batch of agar thus avoiding errors due to growth in media of differing hydrogen ion concentration. The growth was washed off with physiological saline solution and centrifuged, and the sediment, suspended in physiological saline solution containing 0.5 per cent phenol, was adjusted to a fixed density. These suspensions were heated for one hour in a water bath at 55 to 57°C. Details of the titration of these antigens will be given below.

The above method of antigen preparation was chosen because preliminary experiments with heated fourteen-day broth cultures

TABLE 1*

Anticomplementary titration of antigens

QUANTITY TESTED†		NUMBER OF STRAINS	STRAINS
0.9 cc.	0.3 cc.		
++++	++++	3	U, CH, ET
++++	+++	5	D, O, AV, BL, CB
++++	++	6	M, W, W_2, AC, AN, CG
++++	+	5	R, Y, AD, BH, BZ
++++	±	3	AB, AY, CE
++++	−	6	C, AO, BE, CO, DP, EZ
+++	++	2	BK, BL_2
+++	+	2	CF, DA
+++	±	8	B, AE, BA, BC, DB, DS, EX, FE
+++	−	7	H, Z, AU, BF, CU, DO, ES
++	+	2	S, FA
++	±	1	FB
++	−	14	E, V, AF, AW, AZ, BB, BD, BS, CZ, DR, DT, DU, EA, EY
+	±	2	G, FD
+	−	13	A, F, K, AA, AR, AT, AZ_2, BW, CY, DN, EM FF, FH
±	±	2	DK, EE
±	−	33	L, P, X, AM, AP, AS, BM, BP, BY, CD, CL, CM, CN, CS, DC, DE, DL, DM, DV, DX, DY, DZ, EB, EC, ED, EF, EN, EO, EP, EU, EV, EW, FG
−	−	30	N, T, AH, AK, AL, BG, BN, BO, BT, BU, BV, BX, CA, CC, CK, CR, CT, CW, CX, DD, DF, DG, DH, DW, EG, EH, EK, EL, FC, FK

* ++++ = Complete inhibition of hemolysis.
+++ = Marked inhibition of hemolysis.
++ = Moderate inhibition of hemolysis.
+ = Slight inhibition of hemolysis.
± = Very slight inhibition of hemolysis.
− = No inhibition of hemolysis.

† Quantity of 1:30 dilution of stock antigen suspension tested.

(Matsumoto, 1920) and antiformin extracts, as recommeded by Krumwiede and Noble (1918) for precipitin tests, had failed to give satisfactory reactions. Moreover, although Zinsser (1918)

states that bacterial extracts are preferable to suspension as antigens, Kolmer (1923) recommends the latter as being more easily prepared and Zinsser says that they may be used if they have been carefully titrated.

The sera were monovalent rabbit sera, unheated, and obtained usually on the day before use. The rabbits were injected every seven to ten days with 0.5 or 1 cc. of a diluted formalinized broth culture. Bleedings were made after a course of three to five injections, inasmuch as Hooker (1916–1917), and Andrewes and Inman (1919) claim that such sera differentiate more clearly between closely related strains than do those obtained by a longer course of injections.

The complement was the pooled serum of several guinea pigs. One-half cubic centimeter of a 1:20 dilution was used, a quantity representing 1½ to 2 units.

The amboceptor was rabbit anti-sheep cell serum. The unit was determined at the beginning of each day's experiments, and one and one-half times this unit was employed in the tests.

For the red blood cell suspension, sheep's corpuscles were employed. These were washed four times with physiological saline solution and then diluted to 20 times the volume of the original defibrinated blood. Of this suspension 0.5 cc. quantities were used.

EXPERIMENTAL DATA

Preliminary experiments had shown that 0.3 cc. of a 1:30 dilution of the stock antigen suspension was enough to give complete fixation of complement when tested with a suitable serum. For this reason all antigens were tested in 0.9 and 0.3 cc. quantities for anticomplementary activity. The result of this titration is summarized in table 1.

About two months later when it was desired to study more antigens, those which had been too anticomplementary for use at the first test were tested again. The results in the two series are shown in table 2; and demonstrate that on the whole the antigens do not become more anticomplementary with age, but on

TABLE 2*

Repeated anticomplementary titration of antigens

	FIRST TEST		SECOND TEST		
	0.9 cc.	0.3 cc.	0.9 cc.	0.3 cc.	0.1 cc.
B	+++	±	−	−	−
C	++++	−	−	−	−
D	++++	+++	−	−	−
G	+	±	−	−	−
H	+++	−	−	−	−
K	+	−	+	±	−
M	++++	++	++++	+++	−
O	++++	+++	++++	+++	±
S	++	+	+++	±	−
U	++++	++++	++++	++++	−
W	++++	++	−	−	−
Y	++++	+	−	−	−
Z	+++	−	−	−	−
AB	++++	±	+	−	−
AC	++++	++	++++	+	−
AD	++++	+	+	−	−
AE	+++	±	+++	+	−
AN	++++	++	−	−	−
AO	++++	−	+	−	−
AU	+++	−	−	−	−
AY	++++	±	−	−	−
BA	+++	±	+++	−	−
BC	+++	±	−	−	−
BD	++	−	+	−	−
BE	++++	−	++++	++	−
BF	+++	−	+	−	−
BH	++++	+	−	−	−
BK	+++	++	+	−	−
BL	++++	+++	++++	−	−
BL_4	+++	++	++++	+	−
BZ	++++	+	++++	+++	−
CB	++++	+++	++++	++	−
CE	+++	±	++++	++	−
CF	++±	+	++++	+++	−
CG	++++	++	++++	++++	−
CO	++++	−	++±	−	−
CU	+++	−	++++	++	−
DA	+++	+	++	−	−
DB	+++	±	+	−	−
DK	±	±	−	−	−
DO	+++	−	++±	−	−

* For interpretation of table, see foot-note for table 1.

TABLE 2—*Continued*

	FIRST TEST		SECOND TEST		
	0.9 cc.	0.3 cc.	0.9 cc.	0.3 cc.	0.1 cc.
DP	++++	−	+++	+	−
DS	+++	±	−	−	−
DT	++	−	++++	++++	++
EE	±	±	−	−	−
ES	+++	−	++	−	−
ET	++++	++++	++++	−	−
EX	+++	±	++	−	−
EZ	++++	−	−	−	−
FA	++	+	++	−	−
FB	++	±	−	−	−
FD	+	±	−	−	−
FE	+++	±	++	±	−

the contrary, remain the same or become less anticomplementary. The actual figures are: 28 were less anticomplementary at the second than at the first test, 10 were more anticomplementary, and 12 remained essentially unchanged.

The sera to be used for any one day's experiments were first tested with the homologous antigen, and the titre of the serum determined. Following this a number of the antigens, including the homologous one, were tested with several quantities of serum. At the time these experiments were performed, the chief interest lay in the reaction of an antigen with three times the quantity of serum necessary to give complete fixation with the homologous antigen, since a reaction of this or greater intensity was counted as positive and weaker reactions as negative. No attempt was made until later, to determine the smallest quantity of antigen which would give such a reaction, nor to determine how much more antigen or serum would give a positive reaction when a negative reaction was found in the quantities used.

A sample protocol shows the nature of the titrations performed.

I. Amboceptor titration

	TUBE 1	TUBE 2	TUBE 3	TUBE 4	TUBE 5
Amboceptor (1:1000 dilution)	0.5	0.4	0.3	0.2	0.1
Complement (1:20)	0.5	0.5	0.5	0.5	0.5
Red cell suspension	0.5	0.5	0.5	0.5	0.5
Saline	1.0	1.1	1.2	1.3	1.4
Reading (after twenty minutes incubation)	Complete hemolysis	Complete hemolysis	Complete hemolysis	Complete hemolysis	Complete hemolysis

II. Serum titration

	TUBE 1	TUBE 2	TUBE 3	TUBE 4	TUBE 5	TUBE 6	TUBE 7	TUBE 8	TUBE 9	TUBE 10	TUBE 11	REMARKS
Antigen EW	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	Incubation for one hour in water bath at 37°C.
Serum EW (dilution 1:300)	0.8	0.6	0.4	0.2	0.1	0.08	0.06	0.04	0.02	0.01		
Complement (1:20)	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	
Saline		0.2	0.4	0.6	0.7	0.7	0.7	0.8	0.8	0.8	0.8	
Amboceptor	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	Incubation for twenty minutes in water bath at 37°C.
Red blood cells	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	
Reading*	4	4	4	4	4	4	4	1	0	0	0	

III

	TUBE 1	TUBE 2	TUBE 3	TUBE 4	TUBE 5	REMARKS
Antigen	0.3	0.3	0.3	0.3		
Serum EW (1:800)	0.8	0.2	0.1		0.3	Incubation for one hour in water bath at 37°C.
Complement	0.5	0.5	0.5	0.5	0.5	
Saline	0.4	0.5	0.6	0.7	0.7	
Amboceptor	0 3	0.3	0.3	0.3	0.3	Incubation for twenty minutes in water bath at 37°C.
Red blood cells	0.5	0.5	0.5	0.5	0.5	

* See footnote for table 3.

The serum titration (II) was repeated for each serum to be used, and the third operation outlined was, of course, repeated for each combination of antigen and serum.

As it was of advantage to test at least two different sera in a single experiment about 50 antigens were selected for the first

TABLE 3*

Complement fixation tests

	SERUM V (DILUTION 1:100)			SERUM RAWLINGS (DILUTION 1:25)				SERUM V (DILUTION 1:100)			SERUM RAWLINGS DILUTION 1:25		
	0.3	0.2	0.1	0.3	0.2	0.1		0.3	0.2	0.1	0.3	0.2	0.1
L	4	4	3	4	4	3	BX	4	4	4	2	1	0
N	4	4	4	4	4	2	BY	4	4	2	4	4	2
P	4	4	4	4	4	2	CA	4	4	4	1	0	0
T	4	4	4	2	1	0	CC	4	4	4	0	0	0
V	4	4	4	1	0	0	CD	4	4	3	4	4	4
X	4	4	3	0	0	0	CH	4	4	4	3	1	0
AH	4	4	4	2	1	0	CM	4	4	4	3	2	0
AK	4	4	3	2	1	0	CN	4	4	3	0	0	0
AL	4	4	4	3	1	0	CS	4	4	2	4	4	4
AM	4	4	3	2	0	0	CW	4	4	3	4	4	3
AP	4	4	4	1	0	0	CX	4	4	4	3	1	1
AS	4	4	3	0	0	0	DC	4	4	3	1	0	0
AV	0	0	0	4	4	4	DD	4	4			0	
BG	4	4	4	1	0	0	DE	4	4	4	0	0	0
BM	4	4	2	2	0	0	DF	4	4	3	2	0	0
BN	4	4	1	0	0	0	DG	4	4	3	1	0	0
BO	4	4	3	0	0	0	DH	3	3	0	4	2	0
BP	4	4	3	4	4	2	DL	4	4	4	0	0	0
BT	4	4	3	0	0	0	DM		4			0	
BU	0	0	0	3	2	0	DV	4	4	4	0	0	0
BV	4	4	3	2	1	0	DW	4	4	4	1	0	0

*4 = Complete inhibition of hemolysis
3 = Marked inhibition of hemolysis
2 = Moderate inhibition of hemolysis
1 = Slight inhibition of hemolysis
0 = No inhibition of hemolysis

series of experiments since this number was as large as could be conveniently employed. None of these antigens had caused more than a ± inhibition of hemolysis in the 0.9 cc. quantity in the anticomplementary titration shown in table 1, and none had

caused any inhibition in the 0.3 cc. quantity. After these antigens had been tested with seven monovalent sera, all of the remaining antigens which had given no inhibition of hemolysis in the 0.3 cc. quantity and not more than ++ in the 0.9 cc. quantity in the anticomplementary titration were tested with the

TABLE 4

Complement fixation tests

	SERUM FK (DILUTION 1:125)			SERUM FC (DILUTION 1:125)				SERUM FK (DILUTION 1:125)			SERUM FC (DILUTION 1:125)		
	0.3	0.2	0.1	0.3	0.2	0.1		0.3	0.2	0.1	0.3	0.2	0.1
L	4	4	4	4	4	4	CM	4	4	2	4	4	4
N	4	4	4	4	4	4	CR	4	4	3		4	
R	4	4	4	4	4	4	CS	4	4	4	4	4	4
T	4	4	4	4	4	4	CX	4	4	4	4	4	4
V	4	4	4	4	4	4	DC	4	4	3	4	4	3
X	4	4	4	4	4	4	DD	4	4	4	4	4	4
AH	4	4	4	4	4	4	DF	4	4	2	4	4	4
AL	4	4	4	4	4	4	DG	4	4	3	4	4	2
AM	4	4	4	4	4	4	DH	4	4	2	4	4	1
AS	4	4	4	4	4	4	DL	4	4	4	4	4	
AV	0	0	0	0	0	0	DW	4	4	3	4	4	4
BM	4	4	3	4	4	3	DX	4	4	3	4	4	4
BO	4	4	4	4	4	4	DY	4	4	3	4	4	3
BP	4	4	4	4	4	4	EW	4	4	4	4	4	4
BU	4	4	4	4	4	4	EY	4	4	3	4	4	4
BV	0	0	0	0	0	0	FC	4	4	3	4	4	3
BX	4	4	2	4	4	4	FF	4	4	4	4	4	4
BY	4	4	4	4	4	4	FG	4	4	3	4	4	4
CA	4	4	3	4	4	3	FH	4	2	1	4	3	0
CH	4	4	4	4	4	4	FK	4	4	2	4	4	3

same seven sera. By this time two additional sera were available and these were tested with all the antigens previously used (89 in the two series). Following this all the remaining antigens (those which had been at first discarded as being too anticomplementary) were tested again for anticomplementary activity (table 2) and those which appeared satisfactory (29 in number) were tested with seven of the nine sera used previously. Two sera were not used since their reactions had been practically identical with those of other sera in the previous tests.

Tables 3 and 4 summarize the results of these experiments, presenting only in part, by typical protocols, the results obtained.

Since these results are difficult of any close interpretation beyond the general conclusion that some sera obviously react with more antigens than do others, all the findings were reduced

TABLE 5

Complement fixation reactions

ANTIGENS	SERA								
	R	V	AV	CH	DW	EW	FC	FK	Rawlings
A	+	+	+	+	+	+	+	+	+
L	+	+	+	+	+	+	+	+	+
N	+	+	+	+	+	+	+	+	+
R	+	+	+	+	+	+	+	+	+
V	+	+	−	+	+	+	+	+	−
AH	−	+	−	+	+	+	+	+	−
AS	−	+	−	+	+	+	+	+	−
AV	−	−	+	−	−	−	−	−	+
BN	−	+	−	−	+	+	+	+	−
BP	+	+	+	−	+	+	+	+	+
BU	−	−	−	−	−	+	+	+	−
BV	−	+	−	−	−	−	−	−	−
BY	+	+	+	−	+	+	+	+	+
CC	−	+	−	−	+	+	+	+	−
CH	+	+	−	+	+	+	+	+	−
DG	−	+	−	−	−	+	+	+	−
DH	−	+	−	−	+	−	+	+	−
DL	−	+	−	+	+	+	+	+	−
DN	+	+	+	−	+	+	+	+	+
DV	−	+	−	−	+	+	+	+	−
DW	−	+	−	−	+	+	+	+	−
EA	+	+	+	+	+	+	+	+	+
EB	+	+	+	−	+	+	+	+	−
EG	+	+	−	−	+	+	+	+	−
EL	+	+	−	−	+	+	+	+	−
EW	+	+	+	+	+	+	+	+	+
FC	+	+	−	−	+	+	+	+	−
FF	+	+	+	+	+	+	+	+	−
FK	+	+	−	−	+	+	+	+	−

to positive or negative according to Hooker's criterion—an antigen being counted as giving a positive reaction if it shows complete fixation with a quantity of serum not more than three times as large as the quantity which will give complete fixation with the

antigen homologous for that serum. In table 5 are given representative results after they have been converted into positive or negative reactions according to this definition.

The reactions of the remaining antigens are included in table 10. The further interpretation of these findings will be considered below.

Whether or not the results would have been materially different if larger quantities of antigen had been used is partly answered

TABLE 6

Complement fixation tests

Serum AV

ANTIGEN	ANTIGEN							
	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3
	SERUM (DILUTION 1:75)				SERUM (DILUTION 1:225)			
	0.5	0.4	0.3	0.2	0.4	0.2	0.1	0.0
R		4	4	4	4	3	1	0
BV	4	4	4	4	4	3		0
CH	3	2	1	0	0	0	0	0
CR	3	2	1	1	0	0	0	0
DG	2	1	0	0	0	0	0	0
DH	4	4	4	4	3	0	0	0
DW	2	1	0	0	0	0	0	0
EW		4	4	4	4	2	0	0
FF		2	0	0	0	0	0	0
FF-S		3	2	1	0	0	0	0
FK-W	2	2	1	0	0	0	0	0
FK-P	2	2	1	0	0	0	0	0

by the fact that all the antigens tested gave a positive reaction with at least one serum, and all but four with at least five sera. Some experiments conducted to determine this point may also be cited, which show that 0.3 cc. of antigen was at least 2 to 6 times as large as the antigenic unit (table 6). It also appears likely from these experiments that if enough serum and enough antigen could be used, complete fixation of complement might be secured with almost any typhoid antigen-antibody combination.

In connection with this the recent paper of Parker (1923) raises a point which is of considerable importance and which was not taken into account in the experimental work presented above. Starting from a statement of Dean's that there is probably an optimum quantity of antigen and antibody, and that an excess of either may change a positive to a negative reaction as readily as would an insufficiency, she has shown that Dean's idea is true. She found that when various quantities of antigen are tested with various quantities of sera, it is quite possible to obtain positive reactions with large amounts of antigen and serum or with small amounts of antigen and serum, but not with too large quantities of either one and too small quantities of the other. The experiments of Parker were performed with "partial residue" antigens (Parker, 1923; Zinsser and Parker, 1923) while Dean's antigens were the bacterial extracts mentioned previously, and it is possibly on this account that Dean's results were less definite than those of Parker.

No experiments were carried out with *Bact. typhosum* to show that if a number of antigens were made from the same culture, all would react identically with a given serum. Some evidence that they would so react is, however, given by an examination (table 7) of a number of strains of *Bact. paratyphosum B* isolated during a food-poisoning epidemic studied in November, 1922 (Winslow, Hiscock, Rogers and Robinson, 1923). In this epidemic a number of students were infected at the same time by eating egg-salad. From the feces of 9 of them, *Bact. paratyphosum B* was recovered. Antigens were made from these strains and also from a stock paratyphoid B strain and tested by the complement-fixation reaction. All of the epidemic strains reacted alike in this test although they had shown some differences in agglutinability.

Some time after the experiments with typhoid antigen had been completed, it was suggested that the differences in complement-fixing power found might be associated with differences in the hydrogen-ion concentration of the antigens used. As the original antigens had been discarded, a dozen strains were selected at

TABLE 7

Complement fixation tests—paratyphoid B strains from food poisoning epidemic

ANTIGENS (0.2 cc., 1:30 DILUTIONS)	SERUM KB31* (1:200 DILUTION)					SERUM 16A* (1:75 DILUTION)				
	1.0	0.6	0.3	0.2	0.1	1.0	0.6	0.3	0.2	0.1
1A	4	4	4	4	1	4	4	4	4	4
4B	4	4	4	4	2	4	4	4	4	4
5B1	4	4	4	3	1	4	4	4	4	3
5B2	4	4	4	4	1	4	4	4	4	4
9A	4	4	4	4	2	4	4	4	4	4
13A	4	4	4	4	1	4	4	4	4	4
14A	4	4	4	4	3	4	4	4	4	4
16A	4	4	4	4	2	4	4	4	4	3
19A	4	4	4	4	1	4	4	4	4	3
20A1	4	4	4	4	1	4	4	4	4	3
20B1	4	4	4	4	1	4	4	4	4	3
KB31*	4	4	4	4	1	4	4	4	4	3
Enteritidis*	0	0	0	0	0	3	0	0	0	0
TyEW*	2	1	0	0	0	1	0	0	0	0

* 1A to 20B1, cultures from food-poisoning epidemic—*Bact. paratyphosum* B.
KB31, stock culture—*Bact. paratyphosum* B
TyEW, stock culture—*Bact. typhosum.*

TABLE 8

Anticomplementary titration

(Quantities in terms of 1.75 dilution)

STRAIN	pH OF STOCK SUSPENSION	3.0	2.1	1.5	1.05	1.0	0.9	0.75	0.7	0.5	0.45	0.3	0.15	0.1
S	6.9					4			4	4		3		2
Z	6.8	0	0	0			0					0		
AC	6.7					4			4	4		3		0
AT	6.8			0	0			0			0		0	
BA	6.8			4	4			4			2		0	
BH	6.8					4			3	1		0		0
CU	6.9			4	3			2			1		0	
DX	6.8	2	0	0			0					0		
EU	6.8	1	0	0			0					0		
FH	6.9	3	0	0			0					0		
GO	6.8	1	0	0			0					0		
161	6.8					3			0	0		0		0

random, no effort being made to choose those which had previously furnished either good or bad antigens. Antigens were prepared from these 12 strains following the original technic, and their hydrogen-ion concentrations determined colorimetrically with brom-thymol-blue as the indicator. The readings are shown in table 8.

Determinations were also made of the anticomplementary activity of the antigens (table 8) and of the hydrogen-ion concentration of antigen-complement mixtures before and after one

TABLE 9

pH of antigen—complement mixtures

ANTIGENS	ANTIGEN	COMPLEMENT	SALINE	pH OF MIXTURE
	cc.	*cc.*	*cc.*	
No antigen—Saline control			5.0	7.5
S	1.0	1.5	2.5	6.7
	0.1	1.5	3.4	6.9
Z	1.0	1.5	2.5	6.7
	0.3	1.5	3.2	6.8
BH	1.0	1.5	2.5	6.7
	0.1	1.5	3.4	6.7
No antigen—Complement control		1.5	3.5	6.7

hour's incubation (table 9). These investigations were not pursued further, for the differences observed were so slight as to be apparently unimportant; at least, they were not so great as those necessary to cause differences in agglutinability.

DISCUSSION

Examination of the foregoing tables shows plainly that under the experimental conditions typhoid antigens differ in their ability to fix complement with a given serum; and also that when combined with other monovalent sera, the results do not parallel those obtained with the first serum. Whether these differences are due to qualitative or merely quantitative factors is a question

which cannot be entirely answered, although the data presented in certain of the tables (No. 6) make it seem likely that most if not all of the differences could be shown by further experiment to be quantitative.

One purpose of this investigation was to show, if possible, the presence of groups among typhoid bacilli, such as have been found

TABLE 10

Summary of complement fixation tests

REACTION IN COMPLEMENT FIXATION EXPERIMENTS	SERA								
	CH	DW	V	FC	FK	EW	R	AV	Rawlings
BV	−	−	+	−	−	−	−	−	−
DH	−	+	+	+	+	−	−	−	−
AM, AP, BM, BN, BX, CA, CC, CM, CN, DC, DE, DF, DV, DW, DX, DY, DZ	−	+	+	+	+	+	−	−	−
CY, EG, EK, EL, FC, FG, FH, FK	−	+	+	+	+	+	+	−	−
AA, CZ, EB, EC	−	+	+	+	+	+	+	+	−
AR, BB, BP, BY, CD, CS, CW, DN, EF	−	+	+	+	+	+	+	+	+
A, E, L, N, P, R, AZ-2, EA, EW, EY	+	+	+	+	+	+	+	+	+
EU, FF	+	+	+	+	+	+	+	+	+
F, T, V, AF, AL, AT, BG, BS, BW, CH, DR, DU, ED, EH, EM, EN, EO, EP, EV	+	+	+	+	+	+	+	−	−
X, AH, AK, AS, BO, BT, CR, CX, DD, DL, DM	+	+	+	+	+	+	−	−	−
DG	−	−	+	+	+	+	−	−	−
BU	−	−	−	+	+	+	−	−	−
AV	−	−	−	−	−	−	−	+	+

for certain other organisms. Hooker, as previously stated, had found three groups by the use of a method similar to the one employed here. As other workers, using agglutination or agglutinin-absorption methods, had found from four to seven groups, it seemed worth while to attempt to confirm one or another of these results. Although the data have been carefully examined from this standpoint, no clearly defined and mutually exclusive

groups can be identified. Certain sera, derived by the inoculation of single strains, are what may be called polyvalent, since they react with practically all of the antigens used. Certain antigens, also, are capable of giving positive reactions with any of the sera. On the other hand, an arrangement (table 10) of results can be made in which the antigens react with a progressively increasing and then decreasing number of sera. In this series all of the antigens can be fitted except one (AV), which was culturally a typical typhoid organism but which was peculiar in that a potent serum was produced with it only by a relatively long series of inoculations.

TABLE 11

ANTIGEN	EW	R	FK	DW	CH	AV	RAWLINGS
EW	+	+	+	+	+	+	+
R	+	+	+	+	+	+	+
FK	+	+	+	+	−	−	−
DW	+	−	+	+	−	−	−
CH	+	+	+	+	+	−	−
AV	−	−	−	−	−	+	+
L (Rawlings)	+	+	+	+	+	+	+

If mutually exclusive groups were present, then antigens belonging to a given group should react only with sera of this group; and, conversely, a serum of a given group should react only with antigens of the same group. Accepting the definition of positive and negative reactions given above, the existence of such groups for *Bact. typhosum* is disproved, as shown in table 11 in which are given the cross reactions between the sera and the antigens homologous for these sera.

Nor is it possible that such groups could have been demonstrated by the application of a different definition of a positive reaction, since certain of the sera (FC, FK, and DW) reacted with almost all of the antigens practically to the limit of the serum-titre.

The data given in table 11 bring up another point brought out by Swift and Kinsella in their study of non-hemolytic streptococci.

They showed that if a given antigen reacted positively with only a few sera, its homologous serum usually reacted with a large number of heterologous antigens. Such is apparently not true of *Bact. typhosum*, as, for example, in the case of strain EW, for which both antigen and serum react with all other sera and antigens.

The key to the question of the antigenic structure of *Bact. typhosum* is apparently to be found in an elaboration of Durham's (1900–1901) hypothesis that a given species consists of a number of antigen components, and that in any individual strain one or more of these components may be lacking. It seems justifiable to modify this slightly by recognizing the importance of quantitative as well as qualitative differences in view of the results shown in table 6 and in Parker's paper (1923).

The diagrammatic arrangement of strains presented in table 10 is not to be considered as a hard and fast classification, but merely as the result of a particular set of experiments. Had positive and negative reactions been divided by a line other than the one employed, or had other antigens or sera been used, very probably the contour of the diagram would have been modified. On the other hand, it seems reasonable to suppose that a more or less similar figure would be obtained in any investigation in which a large number of strains were tested with a sufficiently large number of sera, particularly if a similar method were followed.

SUMMARY AND CONCLUSIONS

By testing 118 strains of *Bact. typhosum* with 9 sera in complement-fixation tests, antigenic differences in these strains are clearly brought out.

The differences found are probably quantitative in nature, and are not of a kind to render possible the demonstration of antigenic groups.

The complement-fixation test, if carried out with appropriate sera, offers a simple method of identifying "inagglutinable" or "atypical" strains of *Bact. typhosum*.

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EXPERIMENTAL METHOD FOR THE CULTIVATION OF MICROBES IN AGAR CYLINDERS IN THE PERITONEAL CAVITY OF ANIMALS[1]

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In recent years great efforts have been made by various investigators to find new paths in research concerned with the nature of various of the so-called invisible viruses. It is still a burning question how we shall be able to afford artificial conditions of growth for these viruses which will enable us to penetrate deeper into their essence.

Briefly speaking it may be said that all the efforts that have been made to cultivate the great majority of microörganisms belonging to this category have been in vain.

In the composition of the culture media most investigators have tried, as far as possible, to imitate the natural conditions of growth within the animal organism; a result of these efforts is, for instance, the intraperitoneal cultivation in collodion sacs.

This method was applied, many years back, for instance by Nocard. Since then, not much has been heard about it until Gates, of the Rockefeller Institute, gave an exhaustive description of the best methods for the preparation of such collodion sacs and their application in the cultivation of bacteria. The interesting results gained in the cultivation of *Bacillus pneumosintes* from cases of influenza can be partly ascribed to this technique.

[1] American readers will be interested to note that the excellent method described by Dr. Ørskov bears certain points of resemblance to that described by Zinsser and Raymond in the Proceedings of the Society for Experimental Biology and Medicine, 1921, xviii, 121, and to the method more recently used by Dochez in the preparation of a serum for the treatment of scarlet fever.—EDITOR.

Considerations quite similar to those leading to the application of collodion sacs have led me to devise the method I am going to describe in the following pages.

In experimenting with the culture of vaccine virus *in vitro*, I, like so many other investigators, constantly met with disappointments. Media of the most varied constitution never produced any growth of vaccine virus, and, in order to try to imitate, as far as possible the natural life conditions of the virus concerned, I attempted to cultivate it, embedded in agar, in the peritoneal cavities of rabbits and guinea-pigs.

The idea underlying this method was that the various substances from the organism might be expected to penetrate into the agar, and thus possibly afford proper conditions of life for the virus. The principal conditions were that the agar should not be dissolved in the peritoneum and that the leucocytes should not penetrate into the agar and destroy the same. Both these conditions soon proved to be fully complied with, the agar will keep quite intact for weeks, even for months, and the leucocytes do not penetrate into it.

In regard to its diffusibility it soon proved that agar is permeable to the chemical substances in the organism, even to a very great extent, this not only obtains for the substances with small molecules but the non-decomposed proteins will also rapidly diffuse into the agar. This latter process may frequently be observed macroscopically, by noting the diffuse red color assumed by the piece of agar due to the hemoglobin that has been diffused; and, moreover, if the agar be heated to boiling point subsequent to twenty-four hours' stay in the peritoneum, it will be seen that it does not melt, but has been transformed into serum agar which coagulates throughout even as far as the center of the agar clump. That substances inside the agar clump will diffuse out of the same is quite obvious, and can be witnessed directly, for instance, by introducing some blood centrally into the agar. In the course of twenty-four hours, the hemoglobin will have diffused all through the agar and out of it (provided of course, that the red blood corpuscles have been subjected to hemolysis).

One would therefore think that the condition of growth under these circumstances would come rather close to the natural ones under which the microörganisms grow in the animal's body, and that, at any rate, the microbes will have access to the same substances in various quantities as they are accustomed to live upon within the animal organisms.

Furthermore, it is possible to make the conditions of growth approximate still more closely to the natural ones by adding tissue or tissue fluids of various kinds to the agar before introduction into the peritoneal cavity.

The details of the technique are best explained by referring to the illustrations. Figure 1 shows a glass tube, preferably of rather thick glass, in which a plug of non-absorbent cotton has been inserted. On this plug rests a small glass mounting[2] whose mission is partly to support the agar and protect it against mechanical molestation in the animal, and partly to enable us to close the agar around the bacteria when these have been inoculated in stab culture (cf. later). The glass mounting consists of two conically pointed pieces of glass tube interconnected by four glass columns (cf. footnote).

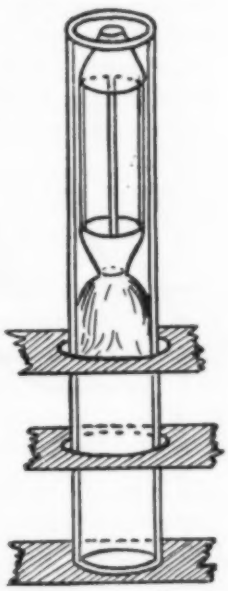

FIG. 1

Glass tube and mounting are packed in and sterilized in the autoclave. After sterilization the glass tube is placed in an ordinary tube-rack, care being taken not to touch the upper part of the outermost glass tube as this will later come in contact with the peritoneum. Melted water agar (2 to 3 per cent agar + salt, carefully washed and filtered) is now poured into the tube, so that the glass mounting is completely covered.

[2] The glass mountings must be manufactured by a glass-worker. Those used by the author have been manufactured by C. C. Jacob, glassware manufacturer, Hauserplads, Copenhagen.

When the agar has congealed, the glass mounting containing it can easily be thrust out of the tube by simply pushing on the cotton plug. The agar is pushed out of the tube to such an extent that it becomes easy, by means of a flame-sterilized knife, to detach the small coat of agar which covers the conical end of the glass mounting. By means of a red-hot metal wire a small depression is formed in the agar, which will facilitate the infusing of the bacteria into the stab culture at a later stage. The inoculation needle[2] with the inoculation material is now introduced into the agar as centrally as possible, the needle being carried between two columns, care being taken not to touch the glass

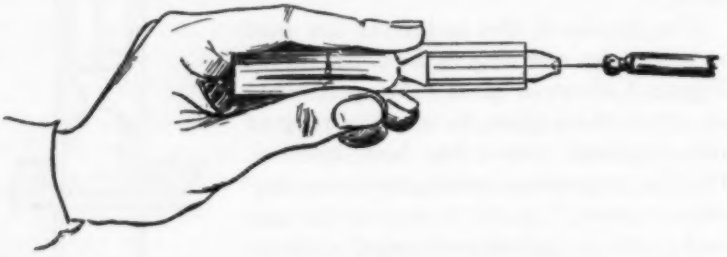

FIG. 2

mounting at any point, as there would thus be free communication into the peritoneal cavity from the stab culture. The inoculation is most easily performed by carrying both glass tube and inoculation needle strictly horizontal, a good illumination being also an essential factor (see figure 2).

Subsequent to inoculation the upper end of the agar should be carefully cemented by carrying a micro burner around the end of the glass mounting, care being taken not to allow the agar to boil too vigorously. After a few minutes cooling, the apparatus is now ready to be introduced into the peritoneal cavity of the animal selected for the experiment. (The size of the apparatus

[2] The inoculation needle is best prepared from a thin platinum wire which is bent double and twisted around a thin needle after which the twisted wire is fixed into the holder. By this means one gets a more elastic inoculation needle and the inoculation material will be better distributed all along the stab.

should, of course, be adapted to the size of the animal to be experimented upon; the apparatus seen in figure 1 would be suitable for a large guinea-pig or a small rabbit.)

If, for instance, our object is to ascertain which bacteria will be developed in the agar within the peritoneal cavity, as compared with the bacteria obtained by cultivation in or on artificial media *in vitro*,—and there is no need to trouble whether the animal possibly gets peritonitis—we need only (prior to the pouring of the agar into the tube) to mix the same with the material containing the bacteria which it is desired to have examined; the same method can be used if one wishes to examine a fluid not containing bacteria, but which is suspected of containing some virus which cannot be cultivated on artificial media.

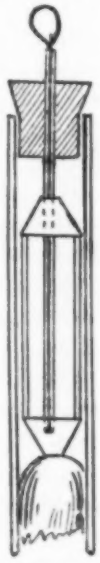

FIG. 3

In certain cases it might be desirable to cultivate the microbes in question in a space filled with a fluid medium within the agar, as for instance in cases in which the microbes concerned are extremely sparsely distributed or are but slightly resistant. If growth takes place at all, one might then get a better chance of demonstrating the microbe in such a small space than if a single small colony formed in the agar, where it may be difficult to detect.

Such a small central canal may be fairly easily prepared in the agar. A hole is burned in a cork, in which is inserted a glass tube so adapted that it can pass through the hole in the conical part of the glass mounting which, according to this method, should not be carried quite up to the upper edge of the outer tube (see fig. 3). The cork serves to fix the central glass tube in the middle of the agar mass in the glass mounting. This central glass tube and the cork should, of course, be sterilized before use. Furthermore, the central tube is provided with a thin metal wire reaching as far as its bottom. The mode of procedure is then as follows: first the melted agar is poured into the tubes so as to cover the glass mounting com-

pletely; then, while the agar is still in a liquid state, the central tube with its metal wire is put down into the agar and fixed by means of the cork so as not to come into contact anywhere with the walls of the glass mounting. When the agar has congealed the metal wire is first removed by careful rotary movements and afterwards the thin glass tube can easily be taken away without damage to the agar. (If one attempts to remove the tube without first having taken away the metal wire, the tube of agar will produce the effect of the piston of a pump and the agar will be damaged.) The small canal thus formed is now easily filled with a fluid medium by means of a minute Pasteur pipette, the point of which can reach quite as far as the bottom of the central space. After filling, the cementing is performed as described above.

The insertion into the peritoneal cavity is performed through a laparotomy opening, the animal being anæsthetized; by carrying the lip of the outer glass tube just inside the opening and by a push at the cotton plug the apparatus is carefully ushered into the peritoneal cavity, where it will settle by itself at a suitable place.

The tissue-fluid will now at once begin to diffuse into the agar and, as early as twelve hours later, a fine growth will, in certain cases, be seen to have taken place in the agar.

In the recovery of the apparatus, all precautions should of course be taken to avoid contamination; the animal is usually first killed. If the agar has been lying only for a few days in the peritoneal cavity, it will usually be seen to be lying rather loosely in the peritoneum, in a fold of the omenta. The longer time it is left in the animal, the more attached will it become to the peritoneum, surrounded by a fine pouch of omenta and intestines. As a rule however, it is fairly easy to cut a small hole through which the agar cylinder can be slipped out.

The detached agar cylinder is now placed in a sterile Petri dish, and, during the examination is best grasped with a surgical forceps so that the teeth each grasp one of the peripheral holes in the glass mounting. With a sterilized knife the outer layer of agar is now removed as far as the glass columns, so as to leave

for examination a cubiform piece of agar bordered by these. By holding the agar against the light the colonies, if any such have formed, may be distinctly perceived.

Further examination can now be carried on under the microscope, perhaps after cutting a cubiform piece of agar from the mounting and placing it on a sterile slide. Occasionally, it may prove practicable to cut the agar into thin slices which can be better examined under the microscope. If the colonies lie crowded "colony-fishing" can be effected by means of the harpoon devised by the author. Transfer from the central canal is most easily performed by means of a Pasteur pipette.

Most bacteria cultured in a central stab within the agar will remain localized only spreading to a slight degree out into the agar, and the cultures can be easily transferred from one stab to another. Thus, Dr. Sigaard Andersen has cultivated the same streptococcus through twelve transfers of agar cylinders without contamination.

The question whether it is possible safely to embed the bacteria within such an agar cylinder may easily be answered in the affirmative by trying, instead of inserting the cylinder into the peritoneum, to place it in a fluid medium. A fine growth will then occur within the stab while the broth remains clear. Most bacterial species will grow excellently in this way, and even markedly anaerobic bacteria which do not grow in broth in an ordinary test tube exposed to the oxygen of the air will show excellent growth as soon as they are embedded in an agar clump, even though this for a time remains swimming on the surface of the broth. I have noticed this, for instance, in experiments with various strains of tetanus bacilli.

Of bacteria that have been examined by this method the following may be named: the colon bacillus, typhoid and paratyphoid bacilli, diphtheria bacilli, tubercle bacilli, cholera vibrios, proteus bacilli, pneumococci, influenza bacilli, streptococci, and several others, all growing, more or less vigorously, in a central stab in the agar.

The cultivation of vaccine virus, for which this method was originally devised, has hitherto not been followed by success.

I tried the method on Levaditi's Neurovaccine, of which a sample was kindly sent to us by the Pasteur Institute in Paris. But even though the neurovaccine was mixed with freshly obtained fetal brain of rabbits we did not succeed in demonstrating any augmentation of the inoculated virus, and, in the third passage, no trace of virus could be demonstrated any longer.

Experiments undertaken in collaboration with Dr. Sigaard Andersen with the object of cultivating filtrable microörganisms, if such exist, from the filtrate obtained from nasal mucus of patients with measles, also gave negative results, inasmuch as we never succeeded in demonstrating any detectable organized microbe. However, I have not yet abandoned these experiments, and by modifying the technique we may perhaps succeed in finding new ways out of the difficulty.

In regard to experiments made with tissue, embedded in the agar, it may be said that whenever the tissue was taken from animals homologous to those into which it was inoculated, it would keep astonishingly well, and, moreover, the author thinks he has seen undoubted signs of growth of tissue, presumably of fibroblasts. Again, the apparently enormous increase in white blood corpuscles which may occasionally be witnessed in an agar stab containing blood from the homologous species of animal might perhaps also be interpreted as a growth and not only as a relative increase in number as seen in proportion to the intact blood corpuscles, which, for that matter, keep well for a lengthy period. It may be that this method will prove to be of advantage also in this respect in certain cases.

In order to examine whether the substances formed in the agar by the bacteria will diffuse out into the organism various species of bacteria, for instance diphtheria and tubercle bacilli were inserted into a series of guinea-pigs.

In the experiments with diphtheria bacilli, undertaken in collaboration with Mr. Schmidt, one of our pharmaceutical chemists, it proved that the animals experimented upon after having had the agar cultures in their peritoneal cavity for twelve days would tolerate up to three times the dose of toxin which was lethal to control guinea-pigs of the same size. In regard

to the experiments with tubercle bacilli, it likewise proved that bacterial products had quite distinctly diffused into the organism; this manifested itself in a reaction to injection of tubercle bacilli deviating from the reaction of normal guinea-pigs. These experiments are too few in number for a more detailed publication, but they will be continued.

Agglutinins were never produced in these experiments, even when there was a vigorous growth of bacteria in the agar (as is the case with colon, typhoid, and paratyphoid bacilli).

The details in the technique to be applied will of course depend upon the nature of the different problems involved. In some cases it may prove of value prior to inoculation to add blood serum or the like substances to the agar.

As compared with the collodion sacs—which probably may show advantages over the agar cylinders in other points—one of the chief merits of the method here described is the greater diffusibility of the agar. As is a well-known fact, non-decomposed proteins will not pass through the collodion membrane; among other things this was manifest in the failure following Gates upon attempts at cultivating Pfeiffer bacilli in collodion sacs, whereas my method gave fine growth of these microbes in twenty-four hours. Furthermore, a solid medium is no doubt frequently preferable to a fluid one, and, finally, I believe that the method described in the preceding pages is easier to control.

STUDIES ON THE BIOLOGY OF SEWAGE DISPOSAL

A SURVEY OF THE BACTERIOLOGICAL FLORA OF A SEWAGE TREATMENT PLANT[1]

MARGARET HOTCHKISS

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INTRODUCTION

Of the many systems providing for sanitary sewage treatment each, in operation, has developed certain disadvantages for which local study and experience have tended to produce local remedies. Those systems which utilize biological factors have excited the interest of many students and the field offers a wealth of problems, upon the solution of which depends the successful operation and control of such sewage disposal plants as contain septic, Imhoff or activated sludge tanks and trickling or contact filter units. As a part of a more extended investigation to determine some of the conditions influencing biological factors a study of the bacterial population of the sewage disposal plant of Plainfield, North Plainfield and Dunellen was begun in November, 1923.

DESCRIPTION OF PLANT

In this plant the sewage passes through a Riensch Wurl screen, Imhoff tanks, sprinkling filter and final settling tank before it is discharged into a small brook. There are six Imhoff tanks, operated in units of three because experience has shown that the digestive efficiency of these tanks is increased by resting periods. After passing through the settling chambers of the Imhoff tanks the sewage, regulated by two dosing tanks, is sprayed upon one

[1] Paper No. 172 of the Journal series, New Jersey Agricultural Experiment Stations and New Jersey State Department of Health, Substation, Sewage Investigations.

of two sprinkling filter beds. The sewage trickles over the stones and drains to a central gallery which leads to the final settling tank. At the time when these experiments were undertaken the effluent from this settling tank was discharged directly into a small brook.

The plant receives three to four million gallons of sewage per day and this represents about the capacity for which it was designed. The sewage is largely domestic waste and street washings are not included. When it reaches the plant it may be termed "stale sewage" as six hours are required for the material to reach the disposal plant after leaving the main sewer where it is collected.

OTHER INVESTIGATIONS

According to general opinion digestion tanks perform their action on sewage by the aid of anaerobic bacteria while aerobic bacteria act most vigorously in contact and trickling filter beds. The Imhoff tanks have for their function the separation and digestion of the solid particles in the incoming sewage and the digested particles form a fine mud which is drawn off on sand beds to dry. The sprinkling filter is designed "to change the putrescible organic matter of the sewage into stable forms, so that the effluent shall not be subject to putrefaction" (Worcester, 1912).

This separation of sewage treatment into two stages seems to have first been suggested by an English sanitary engineer, Scott-Moncrieff when, in 1891, he built a closed tank where anaerobic putrefaction was to take place with a series of trays containing coke for the second stage of nitrification (Metcalf and Eddy, 1916).

Notwithstanding the fact that anaerobic organisms have been considered important in putrefaction Winslow and Belcher (1904) conducting a survey of bacterial flora in stored sewage found few obligate anaerobes at any time. During the time of their experiments they noted a decrease in obligate aerobes while the facultative anaerobes, less sensitive to changes in oxygen tension, became the predominating organisms.

Most of the studies on sewage bacteria have been conducted under aerobic conditions due to the comparative simplicity of technique, and the results show that samples from sewage disposal plants contain an abundance of bacteria which are able to accomplish putrefactive changes under aerobic conditions. Such studies have been reported by Clark and Cage (1905) from the Lawrence Experiment Station, Massachusetts, by Kamm (1917) of the State Water Survey of Illinois and by Gaub (1923) at Rutgers College, New Jersey. The studies at the New Jersey Sewage Substation have not yet included the anaerobes but here also the work has shown an abundance of bacteria producing reduction and oxidation changes under aerobic conditions of culture.

The first work on the bacteria of the Plainfield disposal plant was undertaken in 1922–1923 by W. H. Gaub (1923) a graduate student at Rutgers College. During this investigation numerical determinations of bacteria in raw sewage and effluent of each unit were made daily for a period of a week, this work being done four times at intervals two months apart. Some organisms, able to grow on agar, were isolated and identified. The relative numbers of bacteria effecting certain reduction and oxidation changes were determined. From this work Gaub drew the following conclusions:

1. There was a continual decrease in number of bacteria in each unit of the plant.

2. The effluent of the plant caused an increase of bacteria in the small brook which received it but this increase was entirely eliminated in a distance of 450 feet.

3. Proteolytic bacteria and sulfate reducing bacteria were found in the effluents of all units. Nitrifying bacteria were distributed in Imhoff tank and sprinkling filter effluents with the greatest numbers in the latter effluent. Sulfur oxidizing bacteria were observed to be present especially in the effluents of the sprinkling filter and final settling tank. Cellulose decomposing bacteria were much less numerous than the other organisms studied.

EXPERIMENTAL RESULTS

The present paper represents work which is an amplification of the studies on the prevalence of the various organisms commenced by Gaub. Limitation of personnel and of equipment has prevented a complete and exhaustive study but it is believed that the results obtained may assist in the development of a knowledge of the bacteriology of sewage disposal plants.

The samples examined were collected from the influent to the Imhoff tanks after screening through the Riensch Wurl screen and from the effluent of the Imhoff tank unit. The scum and sludge and also the liquid 4 feet below the scum were examined as representative of the digestion chamber. Since one tank is not in constant operation the figures for this survey are based on data from three different tanks which were in operation at the time of sampling. These samples were collected twice a month for twelve months.

The samples from the sprinkling filter were analyzed once each month. These samples were collected from the surface spray and from the 1-, 3- and 5-foot levels of the bed by means of drain pipes placed for experimental use.

A series of dilutions (1:10, 1:100, 1:1000, 1:10,000, 1:100,000 and 1:1,000,000) was prepared from each sample. Several different media were then inoculated with one cubic centimeter of each dilution and after incubating at 20°C. the cultures were examined and by means of a series of simple chemical tests the dilution which marked the limit of various reactions was determined (Hotchkiss and Murray, 1923; New Jersey, 1923).

The media used to recognize the groups of bacteria were as follows:

1. Bouillon containing a cube of coagulated albumen. This was observed for liquefaction.

2. Bouillon containing ferric ammonium sulfate which was examined for a black precipitate of ferric sulfide as an indication of H_2S formation.

3. Inorganic sulfate medium to detect the formation of H_2S from inorganic material.

4. Thiosulfate medium for oxidation to sulfate.

5. Nitrate solution for the detection of nitrogen gas formation (denitrification).

6. Ammonium sulfate solution for detection of nitrite formation.

7. Nitrite solution for the detection of nitrate formation. It will be observed that 1 and 2 record proteolytic changes, 2, 3, 5 represent reduction processes and 4, 6 and 7 represent oxidation processes. The bacteriological activities studied were therefore concerned with proteolysis and with sulfur and nitrogen transformations. The groups of bacteria producing these changes were universally present and may be assumed to produce similar physiological changes in the sewage during treatment.

TABLE 1

Arithmetical averages. Thousands of bacteria per cubic centimeter throughout sewage plant

BACTERIA	INFLUENT	IMHOFF TANK DIGESTIVE CHAMBER	EFFLUENT	FILTER	FILTER EFFLUENT	FINAL EFFLUENT
Nitrate reducers	278.2	727.7	248.7	208.4	16.2	190.0
H_2S producers (protein)	83.4	424.0	131.9	172.9	2.4	160.1
Albumen digesters	123.9	244.0	60.4	102.6	17.4	135.8
H_2S producers (sulfate)	3.6	215.5	17.0	19.1	0.36	2.4
Ammonium salts to nitrites	43.9	73.0	9.2	39.8	1.3	0.4
Nitrites to nitrates	87.6	83.8	48.3	53.3	12.7	23.4
Thio sulfite to sulfate	2.2	27.8	0.3	2.0	12.8	1.43

The data represented determinations on 20 to 24 samples collected throughout the year from the influent, from various portions of the digestive chamber and from the effluent of the Imhoff tanks. Data on 12 samples each from the various layers of the filter bed was obtained. Data were available on 9 to 11 samples of the final effluent. The maximum number of determinations on the filter effluent was 10, made for the denitrifying organisms and the minimum was 5 determinations made on organisms producing H_2S from protein. The other figures are based on 9 determinations.

The data were first grouped by arithmetical averages as in table 1, which shows the general relationships of the various

bacteria. It is seen that nitrate reducing and proteolytic organisms predominate in the Imhoff tank and sprinkling filter. It is noteworthy that the nitrifying bacteria, which require oxygen and which have been noted as being sensitive to organic material are distributed throughout the plant being present in samples from the Imhoff tank as well as in the sprinkling filter. In the Imhoff tank the nitrifying bacteria do not produce sufficient change to make it possible to detect nitrites or nitrates directly in samples where the bacteria can be demonstrated.

The dilution method of computing bacterial numbers inevitably gives large fluctuations. An attempt to avoid the errors of extreme variation was made by determining the mode of the

TABLE 2

Seven groups of bacteria showing numerical value per cubic centimeter which occurred most frequently

BACTERIA	INFLUENT	DIGESTION CHAMBER	EFFLUENT	FILTER
Nitrate reducers	100,000	1,000,000	100,000	100,000
H_2S producers (protein)	10,000	100,000	10,000	10,000
Albumen digesters	10,000	100,000	1,000	1,000
H_2S producers (sulfate)	1,000	100,000	10,000	10,000
Ammonium salts to nitrites	100	1,000	1,000	1,000
Nitrites to nitrates	0	1,000	0	1,000
Thio sulfite to sulfate	100	10,000	100	1,000

different bacterial groups. This grouping is presented in table 2 and it shows the greatest frequency with which the different groups of bacteria occurred. In it are presented the relative abundance of the bacteria and the general concentration in the digestion chamber of the Imhoff tank as compared with the sprinkling filter is seen. The nitrate reducers are the most numerous in each location, the oxidizing organisms are the least numerous while the proteolytic and hydrogen sulfide producers occupy an intermediate position.

Another statistical arrangement has also been tested, perhaps better fitted to the expression of the figures obtained. This is the geometric average used by Jevons (1883). The geometric average minimizes the effect of occasional large figures. It is

obtained by multiplying the n items of each series and extracting the nth root of the product. Table 3 represents averages obtained by this method using the same data as that on which table 1 is based. A comparison of table 1 and 3 shows the lower figures obtained in the geometric average.

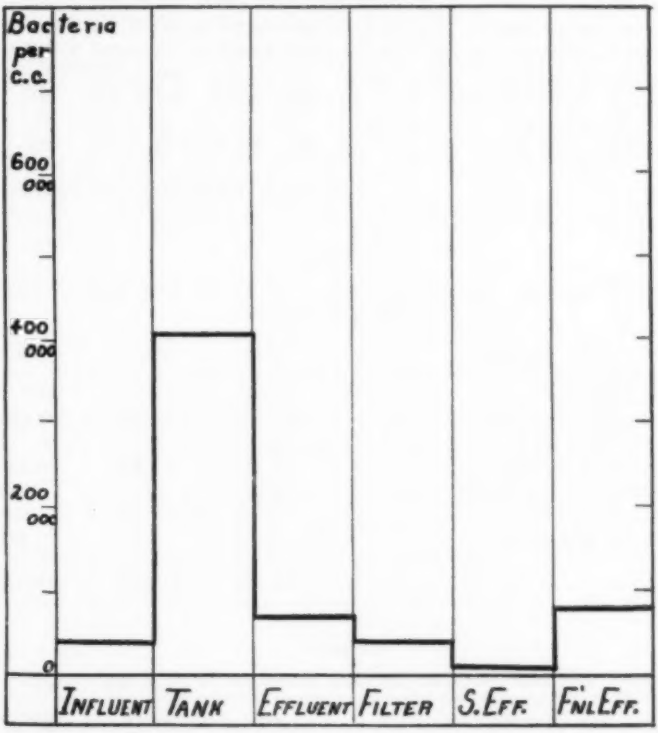

FIG. 1. NUMBER OF BACTERIA, REPRESENTING THE TOTAL OF SEVEN GROUPS, FOUND THROUGHOUT SEWAGE PLANT

The general statements made as to the relative predominance of the organisms are borne out by table 3. All tables show that the digestive chamber of the Imhoff tank contained the greatest concentration of bacteria per cubic centimeter. There were fewer bacteria per cubic centimeter in the effluent from the sprinkling filter than elsewhere in the plant. It must be remem-

TABLE 3

Geometrical averages. Bacteria per cubic centimeter throughout sewage plant

BACTERIA	INFLUENT	IMHOFF TANK DIGESTIVE CHAMBER	EFFLUENT	FILTER	FILTER EFFLUENT	FINAL EFFLUENT
Nitrate reducers	28,480	223,040	64,494	22,456	5,011	74,989
H_2S producers (protein)	10,000	140,154	10,000	9,151	630	5,179
Albumen digesters	3,368	19,419	2,993	9,363	1,930	5,994
H_2S producers (sulfate)	730	21,174	900	1,132	59	100
Ammonium salts to nitrites	332	3,007	367	1,128	59	166
Nitrites to nitrates	74	1,811	22	194	77	215
Thio sulfite to sulfate	316	2,243	53	175	237	158
Total	43,300	410,848	78,829	43,599	8,003	86,801

TABLE 4

Geometrical averages. Bacteria per cubic centimeter in three levels of digestive chamber of Imhoff tank

BACTERIA	SCUM	LIQUID	SLUDGE
Nitrate reducers	480,637	151,999	151,999
H_2S producers (protein)	263,664	87,992	100,000
Albumen digesters	12,452	8,858	59,519
H_2S producers (sulfate)	7,305	15,199	13,688
Ammonium salts to nitrites	6,061	1,492	4,328
Nitrites to nitrates	2,080	592	904
Thio sulfite to sulfate	2,565	299	811
Total	774,764	266,431	341,249

TABLE 5

Geometrical averages. Bacteria per cubic centimeter in three levels of filter bed

BACTERIA	PIPE I	PIPE II	PIPE III
Nitrate reducers	12,338	28,942	28,942
H_2S producers (protein)	16,681	5,994	5,994
Albumen digesters	2,310	3,831	6,812
H_2S producers (sulfate)	681	2,610	837
Ammonium salts to nitrites	383	2,424	1,425
Nitrites to nitrates	68	242	412
Thio sulfite to sulfate	177	209	146
Total	32,638	44,252	44,567

bered that this average would be raised if many samples were collected at the times of spring and fall slough, when the film on the filter stones becomes loosened and passes out with the effluent liquid. It is also noteworthy that after passing through the final settling tank the number of organisms increases. Figure 1 presents these facts graphically.

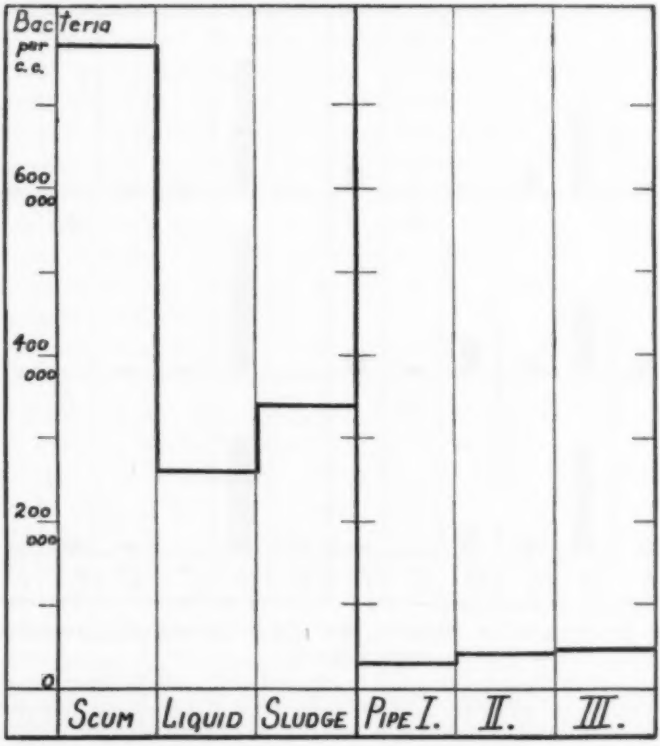

FIG. 2. NUMBER OF BACTERIA, REPRESENTING THE TOTAL OF SEVEN GROUPS, FOUND IN THREE LEVELS OF THE IMHOFF TANK AND THE SPRINKLING FILTER

Tables 4 and 5 show the averages for the data on the samples collected from the digestion chamber of the Imhoff tank and the sprinkling filter bed. The items "scum," "liquid" and "sludge" are used to designate samples taken from the surface layer of the

gas vents, from the material 4 feet below the surface and from material drawn from the sludge pipe which taps the tank at its lower level. The scum is composed of fat, grease and such solid particles as have been brought to the surface attached to gas bubbles, which because of lesser density float on the liquid. Its bacterial content is higher than that of liquid or sludge prob-

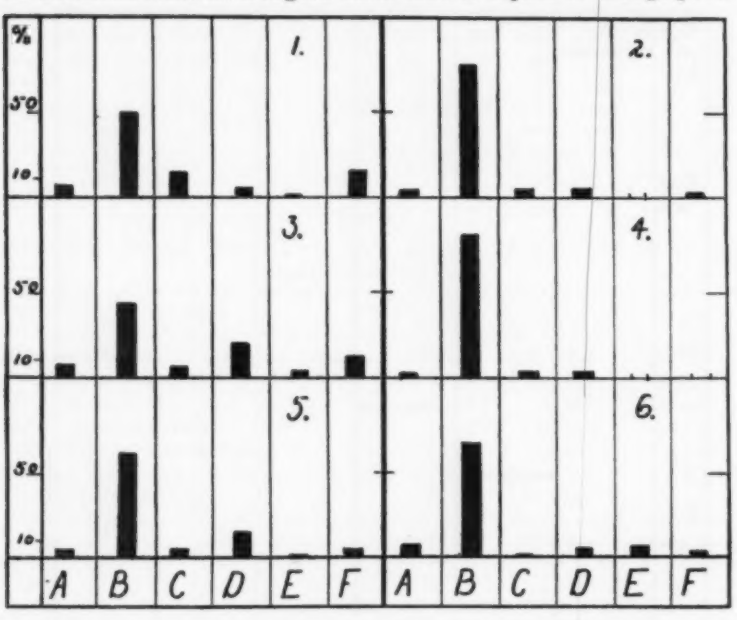

FIG. 3. PERCENTAGE OF BACTERIA PER CUBIC CENTIMETER THROUGHOUT SEWAGE PLANT

A, influent; *B*, digestion chamber; *C*, effluent Imhoff tank; *D*, filter; *E*, sprinkling filter; *F*, final effluent. *1*, nitrate reducers; *2*, H_2S producers (protein) *3*, albumen digesters; *4*, H_2S producers (sulfate); *5*, total nitrifiers; *6*, sulfur oxidizers.

ably for much the same reasons that give cream a higher bacterial count than the milk below it.

The sewage which drains through the filter bed has a smaller concentration of bacteria per cubic centimeter but there is a slight increase in bacterial content at the bottom of the bed (fig. 2). This is probably due to contact with the film which

surrounds the stones and which is the locus for a complex animal and plant population. Bacteriological studies were also made on this film and the results will be presented in a later paper.

Although the digestion chamber contains the greatest number of bacteria per cubic centimeter no striking selective action in the types of bacteria which compose the flora was observed. Figure 3 expresses the percentage of bacteria of each group throughout the sewage plant. Whether oxidizing or reducing bacteria, the highest percentage is found in the digestion chamber. This merely shows the straining action of the tank for as the sewage passes through the settling chamber the solids sink and find their way through the slots into the digestion chamber carrying bacteria with them. Hence the material in the tank becomes more and more concentrated. However, conditions in the tank are not actually hostile to any of these groups of organisms as is witnessed by the fact that the nitrifying and sulfur oxidizing groups are not eliminated.

Does the complexion of the flora change markedly in any of the units of the plant? Figure 4 shows the relative percentage of the groups under consideration in each unit of the sewage system. The bacteria which reduce nitrate to nitrogen gas are most numerous throughout the plant. This is a change which is considered to take place under conditions of insufficient aeration (Marshall, 1921) and it is noteworthy that passage through an Imhoff or settling tank increases the percentage of these organisms. Next to the nitrate reducing organisms in numerical importance are the producers of hydrogen sulfide from protein. These are abundant in the influent, the digestion chamber and the sprinkling filter. Their numbers are diminished as the sewage passes through the plant until they make up about 7 per cent in the filter effluent. This low percentage is not influenced by the passage of the sewage through the final settling tank.

Another numerous group of organisms is the group of albumen digesters. It might be supposed that this group of proteolytic organisms would be most abundant in the Imhoff tank where protein digestion presumably takes place but rather contrary to expectation the percentage is highest in the sprinkling filter. The

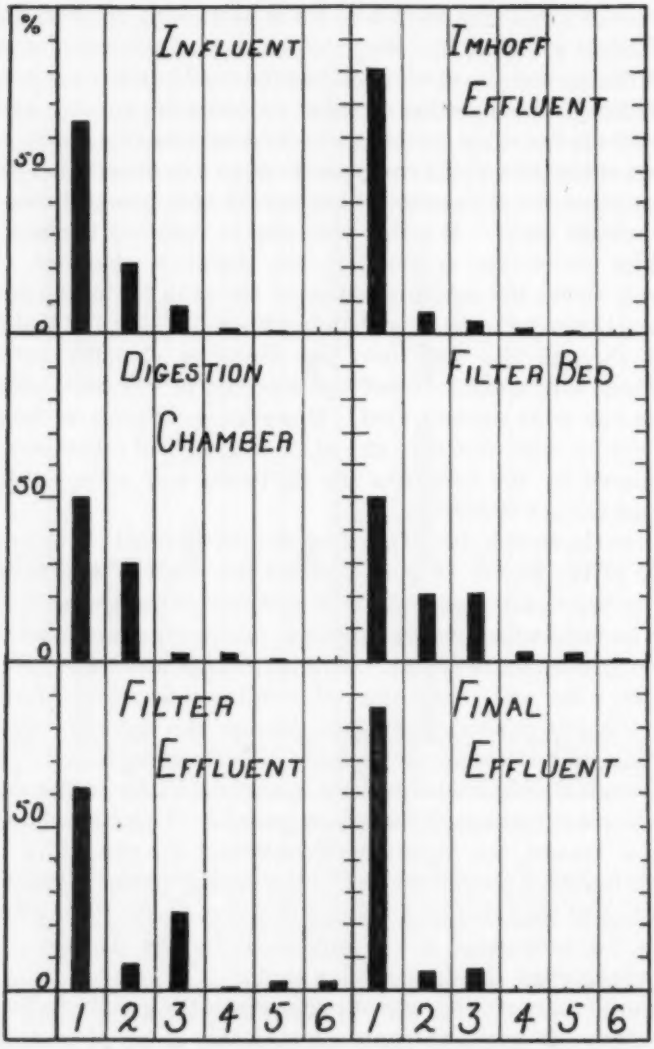

FIG. 4. PERCENTAGE OF BACTERIA PER CUBIC CENTIMETERS IN EACH UNIT OF SEWAGE PLANT

1, nitrate reducers; *2*, H_2S producers (protein); *3*, albumen digesters; *4*, H_2S producers (sulfate); *5*, total nitrifiers; *6*, sulfur oxidizers.

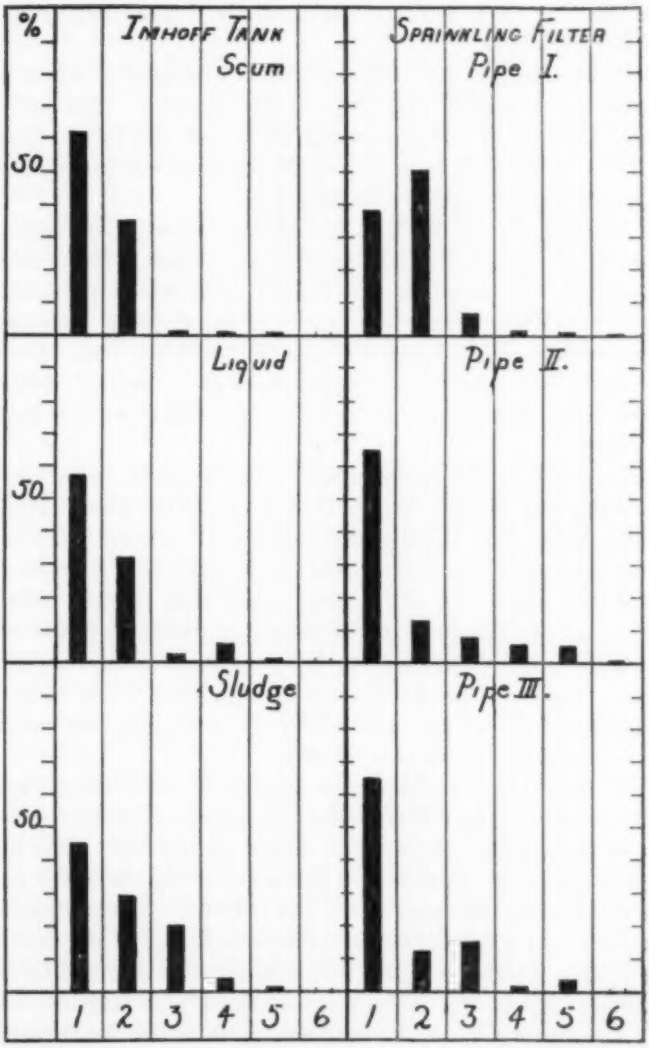

FIG. 5. PERCENTAGE OF BACTERIA PER CUBIC CENTIMETER IN SAMPLES FROM THREE LEVELS OF DIGESTION CHAMBER (IMHOFF TANK) AND SPRINKLING FILTER

1, nitrate reducers; *2*, H_2S producers (protein); *3*, albumen digesters; *4*, H_2S producers (sulfate); *5*, total nitrifiers; *6*, sulfur oxidizers.

actual numbers here average about one-half those found in the digestion chamber.

Both the nitrogen and sulfur oxidizers averaged a barely considerable percentage of organisms in any sample. This percentage was highest in the sprinkling filter and the filter effluent. It is to be noted that even this slight increase was lost after the sewage passed through the settling tank.

No striking change of flora has therefore been demonstrated as the sewage passes through the treatment plant. Some oxidizing bacteria are found in the Imhoff tank which presumably favors anaerobic changes and there is no striking increase in the percentage of oxidizing bacteria in the filter bed. On the other hand throughout the plant there is a decided decrease in the relative percentage of those bacteria which produce hydrogen sulfide.

Figure 5 shows the composition of the material from various parts of the Imhoff tank and the three levels in the filter. It must be remembered that the digestion chamber contains many more bacteria per cubic centimeter than the liquid which finds its way through the filter. In the digestion chamber nitrate reducers and hydrogen sulfide producers predominate slightly in the scum and liquid. Albumen digesters become more numerous in the sludge. In general the proportion of organisms is the same, for the liquid and sludge are agitated by gas evolution and the contents of the chamber become mixed.

In the filter bed a change suggestive of decreasing oxygen tension is seen. The nitrate reducing organisms occupy a larger place as the samples are secured deeper in the bed. The albumen digesters also increase and the numbers in the lower levels seem to influence the character of the effluent. These organisms make up 7 and 8 per cent of the bacteria found in the first and second pipes which are 1 and 3 feet from the surface of the bed and 15 per cent of the bacteria in the third pipe which is 5 feet below the surface while the effluent which drains from the bed contains 24 per cent of these organisms. In contrast to the increase in this type of proteolytic organism in the filter bed it seems that the flow of sewage through an Imhoff or settling tank

does not increase the predominance of the albumen digesters. The organisms form 7 per cent of the influent and 4 per cent of the effluent of the Imhoff tank. They have increased to 24 per cent in the filter effluent and yet the final effluent contains only 6 per cent. Perhaps these organisms find their most favorable habitat around undissolved protein material which tends to sink and so to rid the liquid of the proteolytic organisms.

The filter bed has been regarded as the site of oxidation changes which tend to render the effluent stable and inoffensive. If the sulfur cycle is considered it will be noted that the producers of hydrogen sulfide from protein which form 51 per cent of the bacteria in the filter influent from the top layer of the filter bed decrease until there remain only 7 per cent in the filter effluent. The group of organisms which produce hydrogen sulfide from inorganic sulfates are less numerous. They form 2 per cent of the bacteria in the liquid of the upper level of the filter bed, increase to 5 per cent in the middle layer, decrease to 1.8 per cent in the lower layer while the filter effluent contains 0.7 per cent. In other words these hydrogen sulfide producers tend to be eliminated also. Turning to the sulfur *oxidizing* bacteria, those which grew under our cultural conditions formed only 0.5 to 0.3 per cent of the bacteria in the liquid from the filter bed; however, the effluent from the filter bed contains 3 per cent and this indicates an increase of these organisms. It is, then, the tendency of hydrogen sulfide producers in the filter bed to decrease in numbers but the sulfur oxidizing bacteria increase. In spite of such a tendency the oxidizers do not increase to such an extent that they become of great numerical importance.

In considering the nitrogen cycle it will be remembered that the nitrifying organisms are divided into two groups—those which produce nitrite from ammonium salts and those which utilize the nitrite and form nitrates in so doing. The nitrite producers are here the more numerous group. They form 1 per cent of the organisms in the liquid from the upper layer of the filter bed, 5 per cent in the second layer, 3 per cent in the third layer and 0.7 per cent in the effluent. The nitrate producers form only 0.2 per cent of the bacteria in the liquid from the upper layer,

0.4 per cent in the second layer and 0.9 per cent in the third layer. The effluent contains 1 per cent.

Chemical analyses made by Rudolfs and Campbell (New Jersey, 1923) during the same period show the same story. As table 6 shows, both the nitrate and the nitrite nitrogen increase as the lower levels of the filter bed are reached. Other workers have noted that the depth of the filter bed increases the nitrate nitrogen (Kinnicutt, Winslow and Pratt, 1919).

In spite of the increase in nitrate nitrogen in the filter bed our bacteriological studies show that the nitrate reducing bacteria are predominant. These organisms form 50 per cent of the bacteria in a cubic centimeter of filter liquid, while the nitrifying

TABLE 6

Average nitrite and nitrate nitrogen in liquid from different levels of the sprinkling filter bed, expressed in parts per million (August, 1922, to June, 1923)*

FILTER BED	NO_2-N	NO_3-N
Surface	0.133	0.511
First pipe	0.130	0.494
Second pipe	0.191	1.400
Third pipe	0.213	3.901
Effluent	0.280	5.665

*Compiled from table 13, Bulletin 390, New Jersey Agricultural Experiment Station.

organisms form 3 per cent of the bacterial population in the same location. The filter effluent contains 62 per cent nitrate reducers and 1.7 per cent nitrifiers. The liquid from the digestion chamber contains 57 per cent of nitrate reducers and 0.78 per cent of nitrifiers. The ratio of the nitrifiers to the nitrate reducers in the three locations is 0.059, 0.027, 0.013. Therefore it seems that conditions in the filter bed are such that the nitrifying organisms multiply more rapidly than in other parts of the disposal plant. It has been suggested (New Jersey, 1923) that one factor is the partial digestion of organic matter to simpler forms which are more readily attacked by the nitrifying organisms. This may account for the fact that nitrification is greater in the lower levels of the bed although a priori it would seem that oxygen tension deeper in the bed would be less favorable for oxidation changes.

SUMMARY

By the use of the dilution method using different media the bacterial population in the sewage disposal plant under consideration was divided into arbitrary groups according to physiological activities produced. The groups here discussed are

1. Organisms reducing nitrate to nitrogen gas.
2. Organisms splitting protein with the production of hydrogen sulfide.
3. Organisms liquefying coagulated egg albumin.
4. Organisms reducing inorganic sulfates to hydrogen sulfide.
5. Organisms oxidizing ammonium salts to nitrites.
6. Organisms oxidizing nitrites to nitrates.
7. Organisms oxidizing thio sulfite to sulfate.

In this preliminary survey of the bacterial population no attempt was made to isolate and observe individual bacterial types.

The study was carried on for one year and conclusions are based on the geometrical averages of the data obtained.

The largest number of bacteria per cubic centimeter were found in the digestive chamber of the Imhoff tank. This was true whether organisms of a reducing or an oxidizing type were considered. The effluent from the sprinkling filter, except at the time of slough, contained the fewest bacteria per cubic centimeter.

Of the groups considered, the organisms most important numerically throughout the plant were the nitrate reducers, the hydrogen sulfide producers (from protein) and the albumen digesters. Nitrifying and sulfur oxidizing bacteria occurred throughout the plant, and were consistently found even in the digestion chamber of the Imhoff tank. Chemical analyses of material from the digestion chamber, however, showed no nitrites or nitrates. The number of nitrifying organisms increased in the filter bed although they never became numerically predominant and higher percentages of nitrifiers were found in liquid which had trickled through the lower levels of the bed than that collected near the surface.

An interesting fact was the drop in the numbers of hydrogen sulfide producing organisms as the sewage passed through the plant. This was the most striking change in the bacterial flora which could be demonstrated.

In general it may be said that in a sewage plant handling "stale" sewage in a quantity at about its capacity the proteolytic and reducing organisms overbalance the oxidizing organisms, however the practical results obtained at the plant under examination show that by careful operation a satisfactory effluent can nevertheless be obtained.

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STUDIES ON THE BIOLOGY OF SEWAGE DISPOSAL

A SPRINKLING FILTER BED AND ITS BACTERIOLOGICAL POPULATION[1]

MARGARET HOTCHKISS

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INTRODUCTION

In the course of bacteriological studies made at the sewage substation of the New Jersey Agricultural Experiment Stations and the State Department of Health, an examination of the material which collects around the stones of a sprinkling filter bed was begun. Work was carried on from November, 1922, until November, 1923, and the sprinkling filter bed at the joint sewage disposal plant of Plainfield, North Plainfield and Dunellen supplied the experimental material. A pit for experimental purposes has been sunk in this filter bed and on one side of the pit three holes have been screened off at 1-, 3- and 5-foot depths from the surface. Bricks have been placed in these holes and the material which collected on these was scraped off at weekly intervals. Once a month a bacteriological examination of these scrapings was made.

The bacteriological work was conducted in the same manner as in the previously reported studies (Hotchkiss and Murray, 1923; Rudolfs et al., 1923) and the work given here is an amplification of the investigations on certain bacteriological activities of a physiological nature. These activities were: the reduction of nitrates to nitrogen gas, the production of hydrogen sulfide both from protein and sulfate, the digestion of albumen, and the oxidation of ammonium and nitrite compounds to nitrites and nitrates respectively.

[1] Paper No. 179 of the Journal Series New Jersey State Experiment Stations and New Jersey State Department of Health, Substation, Sewage Investigations.

The studies on the sewage at different parts of the treatment plant showed that the most abundant groups of bacteria were those giving reduction and proteolytic changes, the oxidizing organisms being of less numerical importance. The liquid trickling through the lower levels of the filter bed contained more nitrifying bacteria than samples from the upper levels. Chemical analyses made at the same time also showed higher nitrites and nitrates in the liquid obtained from the deeper portions of the bed. The data presented in this paper, dealing as they do with the bacteria in the film itself, give a further picture of the bacterial flora of the sprinkling filter bed.

The sprinkling filter in question occupies 1.78 acres. It is divided into two sections which receive alternately the Imhoff effluent from two dosing tanks. The liquid is sprayed over the surface and trickles through the bed of crushed stones 6 feet deep and is collected in half tile drains which lead to a central gallery. The sprays work in alternation for four minutes and rest for two minutes so that it takes four minutes for the liquid to pass through the bed.

The stones of the filter bed become a nucleus about which a slimy film collects. In the spring and fall the film loosens and is gradually washed from the bed and a new accumulation takes its place. The film is composed of a mass of animal, fungal and bacterial forms. The seasonal succession of protozoa and fungi found in this film has been reported in previous studies from this laboratory. (Crozier, 1923; Crozier and Harris, 1923; Smith et al., 1921–1922.)

EXPERIMENTAL WORK

As a general survey of the entire plant was in progress, it was not possible to take samples from any one unit of the sewage plant at intervals frequent enough to secure weekly variations in the bacterial population. The data collected have been averaged by the geometrical method used by Jevons. These geometric averages are presented in table 1. Contrary to the findings for the sewage as it passes through the treatment plant,

there is no one type of organism which predominates in the filter bed film. Figure 1 shows that around the surface stones where the effluent from the Imhoff tanks (which may be considered to consist of partially digested material) first settles, the group of organisms which reduce inorganic sulfates to hydrogen sulfide are most numerous. In the next two levels where trickling liquid will carry down some of the decomposition products of the filter itself, the allied group which produces hydrogen sulfide from protein is most in evidence; finally, at the 5-foot level the groups of reducing organisms have reached an apparent equilibrium but one group of nitrifying organisms has gained a slight ascendancy. Figure 1 also shows that the reducing bacteria tend to become fewer as the film is collected from deeper in the bed while the oxidizing bacteria increase.

TABLE 1

Bacteria in the film of a sprinkling filter. Geometric averages of bacteria per gram of dry film

	SURFACE	1 FOOT	3 FEET	5 FEET
Nitrate reducers	964,000	174,000	96,200	1,524,700
H_2S producers (protein)	666,100	36,281,000	5,522,200	1,444,500
Albumen digesters	1,177,900	933,000	1,435,000	1,313,300
H_2S producers (sulfate)	2,854,100	272,000	528,200	361,000
Sulfur oxidizers	66,100	632,500	1,130,100	1,444,500
Nitrite producers	39,900	1,205,000	1,394,300	2,335,500
Nitrate producers	4,000	9,000	13,900	311,400

The number of organisms for each group as they are distributed throughout the film at different levels is shown in figure 2. Proteolytic bacteria, as measured by the organisms which digested coagulated egg albumen, have a general distribution without extreme fluctuations. The greatest percentage of organisms which form hydrogen sulfide from protein was found in the first foot of the sprinkling filter bed, after which there was a decided drop in their numbers and only 3 per cent of these forms occurred at the 5-foot level. The curve representing the denitrifying bacteria (organisms which reduce nitrates to nitrogen gas) presents two peaks, one at the surface film where 34

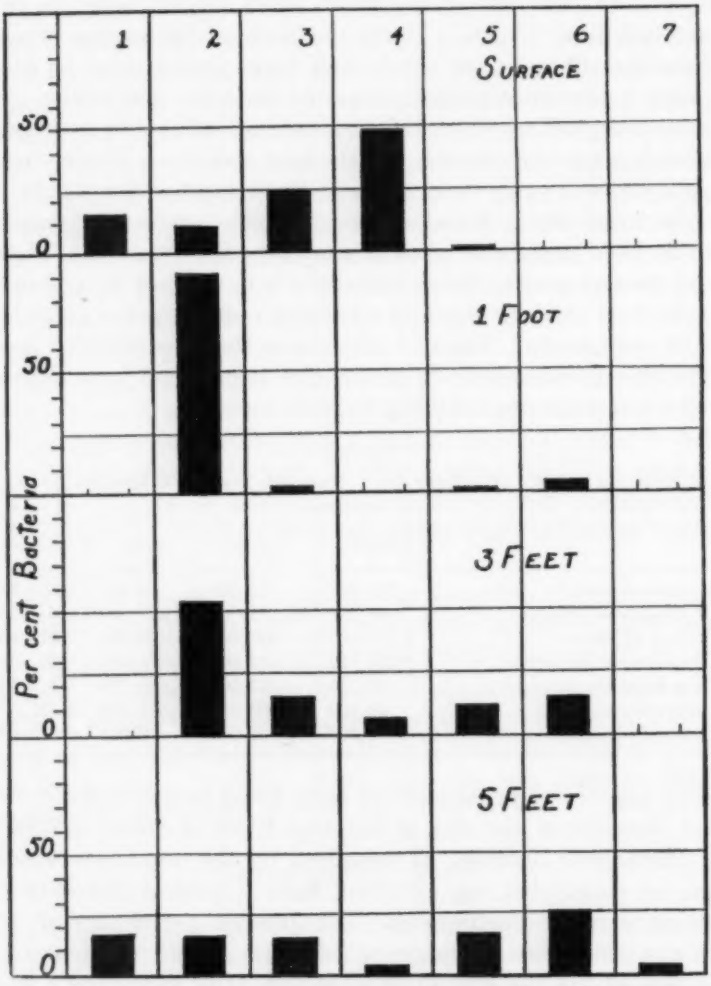

FIG. 1. DISTRIBUTION OF BACTERIA IN THE FILM AT FOUR LEVELS OF THE SPRINKLING FILTER BED (PER CENT BACTERIA PER GRAM OF DRY SOLIDS)

1, nitrate reducers; *2*, H_2S producers (protein); *3*, albumen digesters; *4*, H_2S producers (sulfate); *5*, sulfur oxidizers; *6*, nitrite producers; *7*, nitrate producers.

per cent of the organisms occur and the other in the 5-foot depth where 55 per cent are found. The former site represents the place where the material from the effluent of the Imhoff tank collects and the latter is the spot where the greater portion of the debris from the sprinkling filter itself will be deposited and where also the greatest nitrate production occurs (Rudolfs et al., 1923).

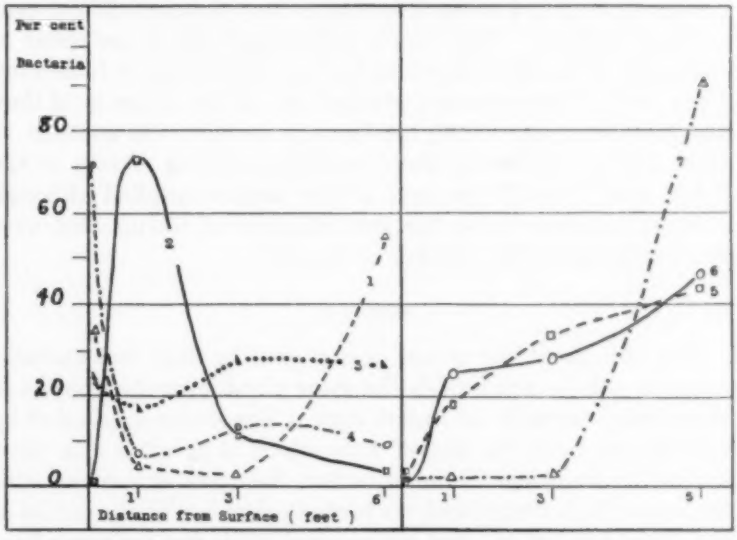

FIG. 2. DISTRIBUTION OF SEVEN GROUPS OF BACTERIA THROUGHOUT FILTER BED (PER CENT BACTERIA PER GRAM OF DRY SOLIDS)

1, nitrate reducers; *2*, H_2S producers (protein); *3*, albumen digesters; *4*, H_2S producers (sulfate); *5*, sulfur oxidizers; *6*, nitrite producers; *7*, nitrate producers.

Although, with the exception of the denitrifying bacteria, the numbers of reducing organisms tend to decrease with the depth of the filter bed, the bacteria effecting oxidation changes increase. While the producers of hydrogen sulfide become less numerous, more sulfur oxidizing bacteria are found (fig. 2). The nitrifying bacteria also show an increase in numbers. The greatest increase in nitrite producing bacteria occurs, apparently, in the first 3 feet of the sprinkling filter bed; while the nitrate

bacteria, being dependant upon nitrite production, increase more rapidly in the lower levels of the bed. Of the nitrite producing bacteria 28 per cent are found at the 3-foot depth and 45 per cent are found at the 5-foot level; 3 per cent of the nitrate producing bacteria are found at the 3-foot level while 92 per cent occur at the 5-foot level. The nitrite producing bacteria represent, at the 3-foot level, 13 per cent of the bacteria in the seven groups studied and at the 5-foot level they represent 26 per cent of these bacteria. The nitrate producing bacteria are never as numerous in the upper levels of the bed for they never form even 1 per cent of the organisms studied and in the 5-foot level they are only 3 per cent of the total groups studied. In contrast to these nitrate producers, those bacteria reducing nitrate at the 5-foot level form 17 per cent of the bacteria studied although chemical analyses show the predominance of nitrification over denitrification in this portion of the bed.

SUMMARY

The film about the stones of a sprinkling filter bed contains bacteria able to accomplish the same physiological activities as those which occur in an Imhoff tank. The stones are seeded by the effluent from the Imhoff tank which is sprayed over their surface. Conditions in the bed affect the types of bacteria with the result that the proteolytic bacteria decrease as the stones of the lower level of the bed are reached while the oxidizing bacteria increase. This increase in oxidizing bacteria confirms the chemical analyses which show a higher nitrate production in the lowest level of the filter bed. Since many bacteria are able to effect denitrification, as measured by the reduction of nitrates to nitrogen, these types of bacteria also may be demonstrated readily in the sprinkling filter film.

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THE CHEMICAL MECHANISM OF BACTERIAL BEHAVIOR

I. BEHAVIOR TOWARD DYES—FACTORS CONTROLLING THE GRAM REACTION[1]

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I. INTRODUCTION

In a recent number of this journal (Stearn and Stearn, 1923) the authors presented some experimental evidence which indicated that the behavior of bacteria toward dyes could be explained on the assumption that it was largely determined by the properties of the bacterial protein, and that this latter is an ampholyte combining with acid dyes on the acid side of its isoelectric point and with basic dyes on the alkaline side. It was suggested at that time that an interpretation of the much discussed Gram reaction might be derived from similar considerations. In the present series of papers the authors are not interested in suggesting any modification of technique in carrying out the staining. Their object is to present data tending to correlate the Gram reaction with the general behavior of bacteria toward dyes, bacteriostasis due to dyes, the significance of the many modifications of technique suggested from time to time, and the fact that in spite of all these improvements over original methods there are yet certain cases of non-specificity toward Gram reaction especially with older cultures.

The clinical importance of this reaction has been well established. This importance can, we believe, be enhanced by technicians in whom, coupled with carefulness in execution, there is an intelligent view of the important factors influencing the reaction and their mutual relationships.

[1] Contribution from the Division of Physical Chemistry and the Public Health Laboratory, University of Missouri.

II

A survey of some of the anomalies discovered now and then in the use of the Gram stain for diagnostic work may be of a great deal of importance especially where these cannot be explained as due to poor stains, old mordant or faulty technique in preparation of slides, and for which explanation must be sought among other uncontrolled factors.

In certain chronic cases Gram-negative organisms such as the gonococcus may hold the primary stain to such an extent that they appear Gram-positive despite the fact that culturally they act as true Gram-negative gonococci (Webster, 1920). Some of the Gram positive organisms when taken up by leucocytes seem to lose their staining characteristics and become Gram-negative. "The bacillus of Friedlander, the diphtheria bacillus, and the *Diplococcus intracellularis* are somewhat variable in their behavior toward Gram's stain, and may or may not decolorize" (Wood, 1909).

According to Czalewski (1896) anomalies arise due to the frequent use of old cultures. Grimme (1902) states that diphtheria bacilli are Gram-positive only in young cultures. In old cultures they may be weakly positive or not stained at all. Mills (1892) found that the Gram character varied somewhat with the composition of the nutrient material on which the organism is grown. Bacilli were found to be Gram-positive when taken from pus but negative when taken from culture media. Schmidt (1892) noticed that *B. coli-communis* and *B. aerogenes* from intestines rich in fat were Gram-positive, while those from intestines low in fat content were Gram-negative. The former remained Gram-positive when cultured.

Wilde (1896) isolated an organism from mice which was definitely Gram-positive but became negative when cultivated. Aronson (1897) found that in young cultures certain Gram-positive organisms acted Gram negative and he attributed this to incomplete fat formation. Burke (1922) has suggested that loss of positiveness in old cultures may be due in part to the formation of acids.

Earlier literature contains many such examples. Later work shows fewer, which may be due to the fact that the source, the habitat, the time of incubation, the nutrient medium of the organism are factors all of which are now well controlled in most laboratories to a greater extent than formerly, and the more uniform methods employed may cover factors previously uncontrolled.

III. EXPERIMENTAL ATTEMPTS AT REVERSING GRAM CHARACTER

Nikitin (1908) states that by rubbing strong acids or alkalies into Gram negative organisms they become positive. Grimme refuted this. Cedercreutz (1908) found that some Gram-negative organisms became positive when treated with fat, starch or egg white. Digestive ferments changed positive organisms to negative. Prolonged treatment with dye, iodine and heat (Neide, 1904) caused Gram-negative organisms to act as positive ones. Eisenberg showed that degenerated cultures which had been Gram-positive in the organ but reacted Gram-negatively could have their original Gram character restored by this method. Heating (Kantorowicz, 1909) or spontaneous degeneration, or the influence of changes in the animal organs (Eisenberg, 1912), cause certain organisms to lose their Gram-positiveness.

IV. PREVIOUS WORK ON THE RELATION OF ACIDITY TO GRAM CHARACTER

Burke (1922) states that "the addition of $NaHCO_3$ results in a greater concentration of methyl violet being present in the Gram-positive organism after decolorization, and lactic acid causes the opposite effect. The failure of Gram-positive organisms from old cultures or from the genital urinary tract to retain the violet dye may be due in part to the presence of certain acids."

Kopeloff and Beerman (1922) advise adding $NaHCO_3$ to the primary stain to neutralize acidity and to improve the intensity of the stain in Gram-positive organisms. They advocate using

an iodine solution to which has been added NaOH since "the free hydroxyl ion may aid in intensifying the stain."

According to Sheppe and Constable (1923) Gram-positive organisms do not retain the primary stain when mordanted with Lugol's iodine solution which has become acid (through light or heat). They found that when $NaHCO_3$ was added to such Lugol's solution normal Gram characters were restored.

Atkins (1920) states that addition of aniline sulphate to gentian violet solution, and of NaOH to iodine, retard decolorization of Gram-positive organisms.

V. EXPERIMENTAL

The experimental data presented fall under two heads: (*A*) The rôle of pH in determining the Gram character of an organism, and (*B*) the function of mordants.

For determining the Gram character of an organism the following modifications of the classic technique were adopted as giving most consistent results: (1) carbol gentian violet was used as primary stain; (2) Lugol's iodine solution was used as mordant; (3) acetone was used as decolorizer. Inasmuch as pure cultures were employed no counterstain was used.

A. The rôle of pH in determining the Gram character of an organism

Table 1 gives data obtained by staining three kinds each of organisms classified as Gram-positive and Gram-negative. The smears were stained two minutes at varying values of pH, mordanted two minutes with the solutions indicated, and decolorized with acetone. No counterstain was used. To insure uniform treatment smears of *B. diphtheriae*, *Bact. typhosum*, *Bact. coli* and *Bact. aerogenes* were made on the same slide. *Streptococcus pyogenes* and *Staphylococcus pyogenes-aureus* were run subsequently.

The significant fact brought out by these data is that any of the organisms, regardless of their nominal Gram character, can be rendered positive to either the basic dye gentian violet or the acid dye acid fuchsin, or negative to either one. The presence or absence of the iodine does not seem to affect the general behavior greatly, as will be brought out more clearly later.

In accordance with the idea that bacteria behave as ampholytes, just as Loeb has shown proteins to behave, all of these organisms in acid solution tend to retain acid dyes. The nominal Gram character is seen to make no difference. In alkaline solution all of these organisms tend to retain the basic gentian violet.

TABLE 1

MORDANTS	GRAM-POSITIVE ORGANISMS			GRAM-NEGATIVE ORGANISMS		
	Streptococcus	Staphylococcus	*B. diphtheriae*	*Bact. coli*	*Bact. aerogenes*	*Bact. typhosum*
Carbol gentian violet used as stain						
HCl, pH = 0.3	neg.	neg.	neg.	neg.	neg.	neg.
HCl plus Lugol's solution, pH = 0.3	pos.	pos.	neg.	neg.	neg.	neg.
HAc, pH = 2.4	pos. (pale)	pos. (pale)	neg.	neg.	neg.	neg.
HAc plus Lugol's solution, pH = 2.4	pos.	pos.	neg.	neg.	neg.	neg.
$NaHCO_3$ plus Lugol's solution, pH = 10.2	pos.	pos.	pos.	pos.	pos.	pos.
NaOH plus Lugol's solution, pH = 11.2	pos.	pos.	pos.	pos.	pos.	pos.
NaOH, pH greater than 11	pos.	pos.	pos.	pos.	pos.	pos.
Carbol acid fuchsin used as stain						
HCl, pH = 0.3	pos. (pale)	pos. (pale)	pos.	pos.	pos.	pos.
HCl plus Lugol's solution, pH = 0.3	pos. (pale)	pos. (pale)	pos.	pos.	pos.	pos.
HAc, pH = 2.4	pos.	pos.	pos.	pos.	pos.	pos.
HAc plus Lugol's solution, pH = 2.4	pos. (very pale)	pos. (very pale)	pos.	pos.	pos.	pos.
$NaHCO_3$ plus Lugol's solution, pH = 10.2	neg.	neg.	neg.	neg.	neg.	neg.
NaOH Lugol's solution, pH = 11.2	neg.	neg.	neg.	neg.	neg.	neg.
NaOH, pH greater than 11	neg.	neg.	neg.	neg.	neg.	neg.

The above results were repeatedly confirmed. All the organisms gave a normal reaction when the usual method of Gram staining was employed.

The only direct experimental data recorded bearing on the above results are presented in a paper by Eisenberg (1912). He found that Gram-negative organisms had a greater tendency

to retain acid dyes, such as cyanosin, than did the Gram-positive ones. Though there was some overlapping, he suggested a possible new classification of cyanosin-positive and cyanosin-negative organisms.

These acid dyes he mordanted with "salts of an acid nature," as for example, alum, picric acid, phenol, salts of the rare heavy metals and even H_2SO_4 at a concentration as high as ten per cent. This latter mordant, besides rendering the stain more firmly fixed,

TABLE 2

Showing the effect of a mixture of carbol gentian violet and carbol acid fuchsin at varying values on pH on both Gram-positive and Gram-negative organism

Time of staining was three minutes. The decolorizer was acetone

pH OF DYE MIXTURE	APPEARANCE OF SMEAR					
	Gram-positive organisms			Gram-negative organisms		
	Streptococcus	Staphylococcus	*B. diphtheriae*	*Bact. typhosum*	*Bact. coli*	*Bact. aerogenes*
3.21	deep pink	deep pink	deep pink	deep pink	deep pink	deep pink
3.47	pale pink	pale pink	pale pink	pale pink	pale pink	pale pink
6.35	pale purplish	pale purplish	very pale purplish	decolorized	decolorized	decolorized
7.3	pale purple	pale purple	pale purple	decolorized	decolorized	decolorized
8.0			pale purple	pale lavender	pale lavender	pale lavender
9.0	pale purplish blue	pale purplish blue	pale purple	pale purple	pale purple	pale purple

The original dye mixture had a pH of 2.6 and in all cases stained all the organisms bright red.

tended to intensify the color, due possibly to sulphonation. These mordants, he states, do not play the same rôle as those ordinarily employed in the Gram reaction.

So far as we have been able to determine, no one has appreciated the significance of these results, and even from his own discussion it is obvious that he himself had little idea of their possible meaning.

In table 1 it will be seen that there is a large jump between the least acid and the least alkaline solutions, i.e., from a pH of 2.4

to one of 10.2. At a pH less than 2.4 all organisms retained the acid dye, and at a pH above 10.2 all retained the basic dye. It would seem that there should be a point, the isoelectric point, at which no dye is retained. Table 2 presents data for this intermediate range.

Slides were stained three minutes with a mixture of basic gentian violet and acid fuchsin buffered at varying values of pH, measured electrolytically. No mordant (other than buffering the dye) was used. The slides were then decolorized with acetone and examined. Confirmation of the facts brought out in table 1 is apparent. Even when both dyes are present the acid dye is retained in acid solution and the basic dye in alkaline solution. In no case was the color as intense as seen in the slides from which table 1 was constructed, due to the fact that each dye was diluted by half on mixing and again by half on adding an equal volume of buffer solution, so that the final effective dye concentration was only one fourth that used in preparing the slides for table 1.

At a pH of 3.47 retention of acid fuchsin is very slight, while, with the exception of the Staphylococcus and Streptococcus, the same is true for gentian violet at a pH of 8.0 or below. Between these limits, or more probably from about 3.8 to 7.5 for the Gram-negative organisms and within narrower limits for the others, there is a distinct range through which little if any dye is retained. This may be thought of for the present as an "isoelectric range." This range has much significance in the theory presented in the following paper and will be discussed there.

There is always a little equivocation in using dye mixtures since one is never exactly sure that the effect of one dye on the other may not have some influence on results. In the above experiments the range through which the organisms seemed completely decolorized might correspond to conditions in which neither dye combines with the protoplasm, or it might represent conditions in which both would tend to combine with it if they did not mutually combine with each other.

To study the behavior of bacteria a little more closely a series of buffer solutions was carefully made up and adjusted to varying values of pH. Instead of using a dye mixture the effect of each

dye was studied independently. The organisms chosen were *Staphylococcus pyogenes-aureus*, a typical strongly Gram-positive organism, *Bact. coli*, a typical Gram-negative one, and *B. diphtheriae*, which is generally listed as Gram-positive but which is known to vary, and which in table 2 is seen to behave in a manner which looks like a "middle course" between the behavior of the Staphylococcus and Streptococcus and of the other organisms.

Smears of all three organisms were made on the same slide and stained. By the clinical technique employed both the Staphylococcus and the *B. diphtheriae* were Gram-positive though the latter reacted rather weakly so. The *Bact. coli* was negative. Slides with the three organisms were then stained three minutes with solutions of carbol gentian violet in one series and with carbol acid fuchsin in another. They were then mordanted for three minutes with the buffer solutions at pH values varying by one unit. The results are indicated in figure 1. The broken lines indicate action toward acid fuchsin, the unbroken ones toward gentian violet. As abscissae are plotted the pH values of the buffering solutions while the ordinates represent intensity of color after decolorizing with acetone on an arbitrary comparative scale.

These curves, it seems to the authors, give a clue to the prime difference in behavior between Gram-positive and Gram-negative organisms. The Staphylococcus is seen to retain the basic dye from a pH of 3 up, while *Bact. coli* only begins to retain it to any extent at a pH of 8.0, and unmordanted *B. diphtheriae* shows but little greater tendency to do so.

Several points of interest are brought out by these curves. It has already been mentioned that Sheppe and Constable (1923) found that addition of hydriodic acid to a pH of about 3 caused typical Gram-positive organisms to react as amphophiles. A glance at the curve for Staphylococcus shows that it retains basic dye to just about this pH, but below this point it tends to become decolorized. The authors (Stearn and Stearn 1923) showed that cultures of *Bact. coli* in media at a pH above 8.0 tended to remove gentian violet from such media, while below this value of pH some of the dye remained in solution.

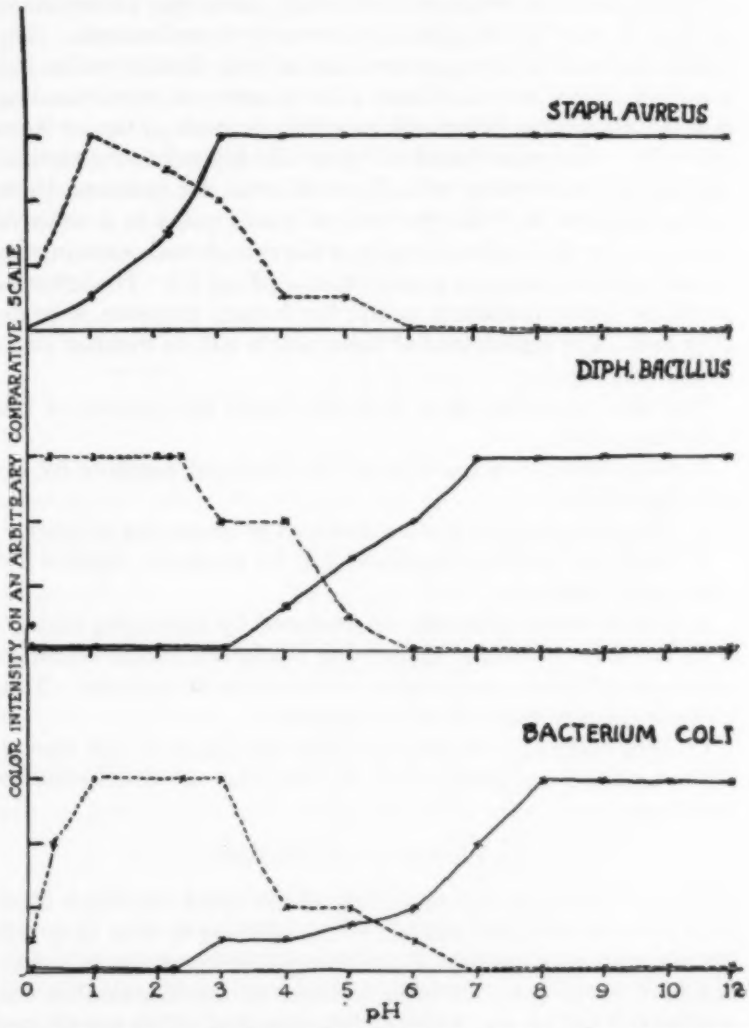

FIG. 1. SHOWING THE RETENTION OF ACID (BROKEN LINE) AND BASIC (UNBROKEN LINE) DYE AT VARYING VALUES OF pH

Discrepancies in measured isoelectric points may be explained at least in part by the behavior shown by these bacteria. The isoelectric point of a pure protein has a fairly definite value, but careful workers have not been able to agree on corresponding points for the same living cells to within as much as two or three pH units. The point found will generally depend on the method employed. In working with *B. diphtheriae*, for instance, these curves indicate that the dye method would point to a value of about 4.5. for the isoelectric point, while cataphoresis experiments would probably indicate a point near a pH of 3.0 The latter is probably a true isoelectric point; the former, however, is probably not. The significance of these points will be touched on in a following paper.

The data presented here have convinced the authors of the following points:

1. Gram-positive organisms can be rendered negative by increasing acidity.
2. The opposite effect can be produced by increasing alkalinity.
3. Acid dye positive organisms can be rendered negative by increasing alkalinity.
4. The opposite effect can be produced by increasing acidity.
5. There is a certain range—the isoelectric range—through which there is little tendency for any stain to be retained. This range is characteristic of each organism.
6. This range has, in general, wider pH limits in the case of Gram-negative organisms than in the case of Gram-positive organisms.

B. The function of mordants

A very significant fact regarding all the mordants which have been recommended for clinical Gram staining is that they are all mild oxidizing agents. Even though the formation of a dye-mordant precipitate has been stressed as the function of the mordant, it will be seen that the following list, which constitutes all of those ever seriously proposed, contains nothing but mild oxidizing agents. Besides bromine and iodine there have been proposed picric acid, trinitrocresol, trinitrobenzoic acid, trinitro-

resorcin, dinitrophenol, dinitrobenzene, trinitrotoluol, trinitroxylol, trinitraniline, orthometa, and para. Even when the iodine is dissolved in tenth normal NaOH as suggested by Atkins (1920), the resulting hypoiodite is still a mild oxidizing agent. In most cases the action of such agents must not be too strong, or the color may be bleached by the destruction of the dye.

Data presented below indicate that not only the above named substances, but practically any other mild oxidizing agents are

TABLE 3

Action of oxidizing agents as mordants

ORGANISM	$K_2Cr_2O_7$	$KMnO_4$	BLEACHING POWDER	NEUTRALIZED H_2O_2	BRUSHES WATER	COMMERCIAL H_2O_2	LUGOL'S IODINE
Gram-positive organisms							
Streptococcus	strong pos.	pos.	pos.	pos.	pos.	weak pos.	pos.
Staphylococcus	strong pos.	pos.	pos.	pos.	pos.	weak pos.	pos.
B. diphtheriae	strong pos.	pos.	pos.	pos.	pos.	weak pos.	pos.
Gram-negative organisms							
Bact. coli	strong pos.	pos.	pos.	weak pos.	weak pos.	weak pos.	neg.
Bact. aerogenes	strong pos.	pos.	pos.	very pale	weak pos.	weak pos.	neg.
Bact. typhosum	strong pos.	pos.	pos.	very pale	weak pos.	weak pos.	neg.

very satisfactory as mordants in Gram staining. These data (table 3) were obtained by staining slides with carbol gentian-violet for two minutes, mordanting two minutes with the various oxidizing agents and decolorizing with acetone. No counterstain was used. Approximately N/50 solutions of the mordants were used as this is about the concentration of Lugol's iodine solution. The solutions are arranged in the table in the probable order of their chemical oxidizing potential, the stronger agents coming

first and the milder ones last. In no case was anything added to change the reaction of the aqueous solution except that of the neutralized hydrogen peroxide, which was neutralized with $NaHCO_3$. The other solutions would give for the most part a nearly neutral or slightly acid reaction, especially bromine water and the commercial hydrogen peroxide.

Potassium dichromate is by far the most effective mordant we have used. Its use is seen to render even the Gram-negative organisms positive, at least so far as retaining stain is concerned. This table further shows that the "general" behavior of both types of organisms is essentially the same. The milder oxidizing agents act selectively on the organisms which tend of themselves to be Gram amphophile, but a more vigorous action affects even such typical Gram-negative organisms as those above listed.

If the mordant action is an oxidation, its effect should be reversed by reducing action. That this is the case is indicated by the following experiment. It was found (table 1) that at a pH of 0.3 a certain culture of *Streptococcus pyogenes* was decolorized. Mordanting with Lugol's solution at this pH caused the Streptococcus to retain the stain. Three smears of this culture were stained and mordanted with Lugol's iodine adjusted to a pH of 0.3 with HCl. Slide 1 decolorized with acetone, retained the stain. Slide 2 was treated with a pure acid solution at a pH of 0.3, while no. 3 was treated for the same length of time (ten minutes) with a saturated solution of stannous chloride at this same pH. Slide 2 retained the stain while No. 3 readily gave up its deep color upon the addition of the first few drops of acetone and was completely decolorized. The stannous chloride, a typical and commonly used reducing agent had counteracted the mordanting effect of the iodine. That its action was not due to the dissolving of any dye-iodine precipitate is evident from the fact that stannous chloride itself precipitates gentian violet.

Thus the subsequent reduction had lessened the affinity of the protein for the basic dye, which affinity the previous oxidation with iodine had strengthened. Reducing agents, when allowed to act on protein which had not been previously oxidized, seemed to have little effect one way or the other on the affinity of the protein for dye.

The chemical effect of oxidation is in general to render the substance oxidized more acidic in character. Thus any affinity for a basic dye is increased. Figure 2 shows the effect of

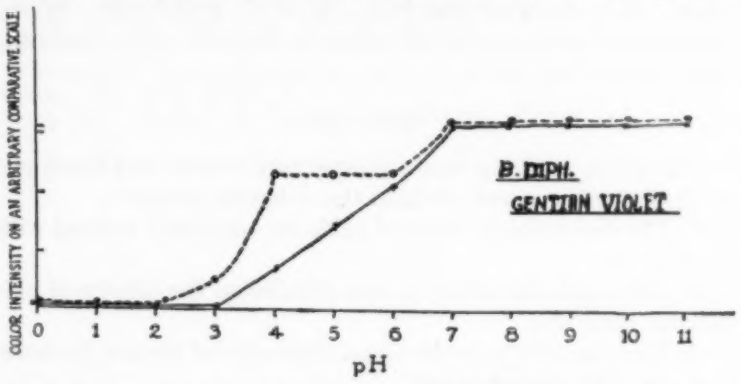

FIG. 2. SHOWING THE EFFECT OF MORDANTING ON THE RETENTION OF BASIC DYE BY AN ORGANISM OF VARIABLE GRAM CHARACTER AT DIFFERENT pH VALUES

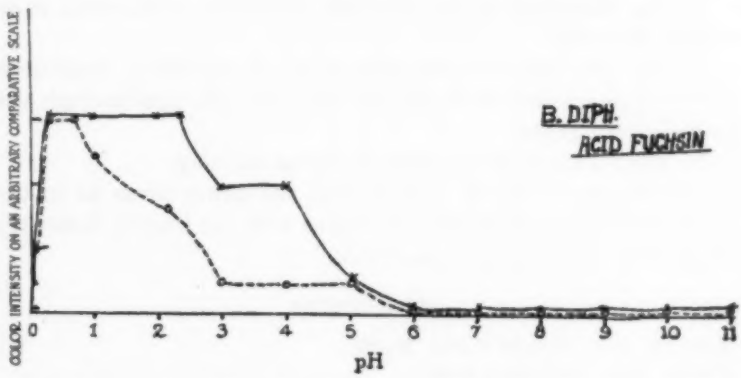

FIG. 3. SHOWING THE EFFECT OF MORDANTING ON THE RETENTION OF ACID DYE BY AN ORGANISM OF VARIABLE GRAM CHARACTER AT DIFFERENT pH VALUES

mordanting *B. diphtheriae*. The coordinates are the same as those in figure 1. The dotted line represents the mordanted, and the unbroken line the unmordanted, smears. The effect is to shift the isoelectric range toward the acid side of the curve.

Naturally the effect of such mordanting action on the affinity of protein for acid dye should be opposite, i.e., at any particular pH this affinity should be decreased. This is shown in figure 3 which also represents the behavior of *B. diphtheriae*. Superposition of figures 2 and 3 will bring out the shift in the isoelectric range mentioned above.

SUMMARY

The theory evolving from our experimental data and developed in the following paper explains the following points:

1. The decolorizing effect of acids on organisms stained with basic dyes.
2. The stabilizing effect of mild alkalies on the affinity of protein for basic dye.
3. The variability of the Gram character of certain bacteria.
4. The function of the pH.
5. Gradations of positiveness in both groups of organisms.
6. The known facts of bacteriostasis.
7. The extension of the artificial variability of bacteria as to Gram character.
8. The somewhat greater affinity of all organisms, regardless of Gram character, for basic dye than for acid dye through the ordinary pH range.
9. The function of mordants in Gram staining.

The theory is also in accord with the newer ideas as to the mechanism of permeability, as well as with the known chemistry of proteins and conjugate proteins.

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THE CHEMICAL MECHANISM OF BACTERIAL BEHAVIOR

II. A NEW THEORY OF THE GRAM REACTION[1]

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I

Throughout the large number of suggested modifications in technique for Gram staining a few conditions have always, whether intentionally or not, been kept constant. The first and most important is the character of the primary stain. Many individual dyes have been suggested, for example pararosaniline and the basic rosaniline dyes in general, pyronine, rhodamine and safranine. All the dyes which give satisfaction—all in the above list—are basic in character. That these are lipoid soluble has been deemed important.

Another condition invariably adhered to is the use of a mild oxidizing agent as mordant. This was noted and discussed in a preceding paper.

Still another observation upon which all workers are agreed is that of the decolorizing power of acids for the primary stain. Most papers do not differentiate between the action of acid on basic and acid dyes. We have shown that acid dye stains are less easily decolorized in the presence of acids than in neutral solution, but, as was stated above, the primary stains used are all basic so that this observation is quite general. In the same way workers are agreed that the addition of mild alkali such as $NaHCO_3$ will render results much more clear cut.

This section may be summed up by stating that good differential results, so far as technique is concerned, can be obtained by

[1] Contribution from the Division of Physical Chemistry and the Public Health Laboratory, University of Missouri.

employing a deep colored basic primary stain, "mordanted" by a mild oxidizing agent in slightly alkaline solution.

II

It is not the function of this section of the paper to propose a definite theory of the Gram reaction, but it seems to the authors that it is quite possible to eliminate one of the two current explanations. Inasmuch as there are more than two possible explanations, elimination of one does not mean sanction of the other without modification.

The two current explanations are the so-called "physical" and the "chemical." It is not clear who suggested the latter explanation, but it has been attributed to Unna who is thought to have made the assertion that "para-rosaniline dyes with iodine and bacterial protein form a relatively insoluble compound." In recent years, however, led perhaps by the colloid chemists, there has been a distinct tendency to shy away from such chemical explanations, and the physical explanation, based on certain postulates as to diffusion and permeability commonly current, seems to be at present the favorite.

According to this idea the dye squeezes through the pores in the cell membrane followed by the mordant molecule, and there, on the inside of the cell, these unite to form a large molecule which cannot squeeze out in the case of Gram-positive organisms. Other organisms either do not permit the dye to enter or else do not hinder the escape of the large dye-mordant molecule. Whatever the explanation of the Gram reaction may be, the above suggestion seems out of the question. It is perhaps best championed by T. H. C. Benians (1920) in a very interesting paper. He divides organisms into three groups: (1) Gram-positive which absorb the dye, (2) Gram-negative which absorb the dye, and (3) Gram-negative (*B. coli*) which do not absorb the dye. The present authors have shown (Stearn and Stearn, 1923) that *Bact. coli* will carry down basic dye when the acidity of the solution has been sufficiently lowered. A striking confirmation of results published in the article referred to above is shown in table 1. The fuchsin was obtained from a bottle labeled "acid fuchsin,"

and there was great surprise that it should behave in just the same way as the basic gentian violet, combining with the *Bact. coli* more in alkaline solution than in acid. This led to the discovery that the dye used contained no trace of sulphur, and thus could not be acid fuchsin, which is a sulphonic acid derivative of ordinary fuchsin. We were dealing with a basic dye which was acting in a perfectly normal manner.

Even a Gram-positive organism, such as *Staphylococcus pyogenes-aureus*, will fail to carry down a basic dye when the solution is sufficiently acid. The following experiment, following the technique by which Benians arrived at the classification given above, shows this.

Experiment. Into a tube containing 2 cc. 1:40,000 parts gentian violet and HCl to a pH of 1.2, twenty-four hours growth of the Staphylococcus was washed, shaken and thrown down in a centri-

TABLE 1

CULTURE	FINAL pH	APPEARANCE OF LIQUID
Bact. coli	9.0	colorless
Bact. coli	8.5	slight pink
Bact. coli	8.45	slight pink
Bact. coli	4.75	deep red

fuge. The liquid remained deeply colored and the bacterial growth was only lightly stained. Another tube with dye at a pH of about 3.0 was partially decolorized by another culture of the same organisms, which organisms in turn became blue in color. At a higher pH (5.5) the bacterial growth practically removed the color from the solution.

Coupled with Benian's results this experiment indicates that any such classification is arbitrary. Under certain conditions Gram-positive bacteria will take up basic dye, under other conditions they will not: under certain conditions Gram-negative bacteria will take up basic dye, while under other conditions they will not. These conditions were not specifically controlled in Benian's work.

The principal criticism of this physical explanation comes, however, from the following considerations:

A. The dye-iodine molecule cannot be of an order of magnitude much larger in size than the dye itself. If we consider the dimensions as molecular and not colloidal, then calculations of molecular volumes will show that it would require either the straight addition of over fourteen atoms of iodine, or the substitution of sixteen atoms for hydrogen atoms to merely double the size of the molecule, assuming the reaction to go in a manner similar to that in which eosin is produced from fluorescein by bromination. That is, the atomic volume of iodine in its compounds is less than 7 per cent of the molecular volume of gentian violet calculated from either Traube's (1899) or Kopp's (1842; 1855) values. Actually there are only twelve possible places where iodine could substitute in this way, and the likelihood of more than four atoms going in is very small. The maximum increase in molecular size, then, would be about 75 per cent, while 20 per cent would very probably more nearly represent it. If it be assumed that the free dye is not molecularly dispersed but in a colloidal state, then it is hard to explain the action of the iodine. Certainly it is not mere coagulation of the colloid, since the precipitate is a product different from the original dye. It may also be pointed out that if such large molecules are really essential, it would be difficult to reconcile many statements that the mordant, though important, is not absolutely necessary, and also that certain substances, e.g., H_2O_2, which we have shown in the preceding paper to have the properties of mordants do not precipitate gentian violet.

We have found in this laboratory that the dye-iodine precipitate, while still basic in character, is less so than the original dye. It was found to be more difficultly soluble in HCl and seemed to be an oxidation product of the original dye since such reducing agents as stannous chloride tended to yield some of the original dye again. The effect of the mordant on the dye seems to be mild oxidation, and it is believed by the authors that this effect is only a secondary matter and of little or no significance in the Gram reaction. The real function of the mordant, it is thought, lies in its action on the bacterial matter. This will be discussed in a subsequent section.

B. In the light of recent researches one can no longer consistently view the process of diffusion through a semi-permeable membrane in the light of a "differential screening" or a "selective filtration." From such a point of view it would be difficult indeed to explain Kahlenberg's (1906; 1921) experiments in which he succeeded in dializing a colloid from a crystalloid through a membrane permeable only to the former. By using pyridine as solvent and vulcanized rubber as membrane one can dialize camphor from silver nitrate, naphthalene from lithium chloride, sulphur from cane sugar, etc. One crystalloid can be separated from another, one colloid from another, a crystalloid from a colloid or colloid from a crystalloid by proper choice of solvent and membrane. There is absolutely no relation, in selective permeability, between size of molecule and diffusion. For example colloidal metallic soaps such as copper oleate can be dialized from lithium chloride, the former going through the membrane.

It has been shown that under pressure (West, 1923) water can be made to pass through glass. One of the authors has shown that this phenomenon also depends on the "membrane" which in this case is the type of glass. A good grade of non-sol glass which is highly insoluble in water will prevent diffusion of water at pressures under which water readily penetrates the more soluble grades of glass found in cheaper ware. In such experiments thickness of glass does not seem important, since the laboratory ware used was quite thin, while the cheaper glass through which water passes more easily is in general thicker.

The significant factor in determining permeability is mutual solubility—not size of particle. With a hydrocarbon membrane such as rubber, materials like naphthalene and camphor, which readily dissolve in it, will pass through, leaving behind salts such as silver nitrate, lithium chloride and other crystalloids like sugar, etc. The mechanism is a matter of solution in the membrane on that side where the concentration is high, and what might be thought of as evaporation from the membrane on the other side into a more dilute environment.

C. Finally, from the physical theory of the Gram reaction one should expect either fairly specific differences, i.e., constancy of

Gram character, or else all organisms should be about equally variable, this variability being measured by a hypothetical elasticity of membrane pores. Czalewski (1896) states that even among Gram-positive organisms there are many gradations of "positiveness." Spirilla as a class are very easily decolorized, Rauschbrand bacillus is less so but is still easily decolorized, while *B. tumescens* is very retentive of the primary stain. There is no very sharp line between the two types of organisms, Gram-positive and Gram-negative, and slight changes in environment—sometimes merely the age of a culture and the effect of change in media due to growth—seem to alter the Gram character.

III. OUTLINE OF THEORY

The authors have shown that much of the behavior of bacteria toward dyes can be explained by assuming that the chemistry of the bacterial protein is the determining factor. It is significant to note how far the same simple assumption will carry one in understanding the mechanism of the Gram reaction.

Much has been made of the lipoid content of cells. Kendall (1916), from some of the data of Eisenberg, goes so far as to state that the Gram character is determined by the lipoidal content of the cell membrane, and specifically by the unsaturated fatty acids, phosphatids, etc. This means to us that bacterial protein, as might be expected, is not a pure simple protein, but a conjugate protein. An example of such would be a lecithoprotein. Such a substance is a loose combination of a simple protein with a phosphatid such as lecithin which would be stable only between certain pH values. The character of simple proteins has been well worked out by Loeb, but little is known of the physical behavior of the phosphatids. Throughout the ordinary pH range they are said to act as acids and they are much more distinctly acid than are the proteins. Like the proteins they are, however, amphoteric, and can exist in the form of such salts as lecithin chloride in sufficiently acid solution, as well as metallic lecithinates in more nearly neutral or alkaline solutions. Little if any work has been done to determine the isoelectric points of these substances, but their distinctly acidic character

would throw such points at a fairly low pH. Even nucleic acid, which is more acidic by far than the phosphatids, is itself an ampholyte having an isoelectric point at a hydrogen ion concentration of about 2×10^{-1}, or a pH of 0.7 (Fodor, 1920). There is reason to believe, from recent work of Winslow, Falk and Caulfield (1923), that for certain phosphatids this point comes in the neighborhood of a pH of 3. This will be discussed later.

Upon the above assumption of a conjugated protein the predicted behavior of bacteria is as follows:

1. In very acid solutions, whose reaction is on the acid side of the isoelectric points of both protein and phosphatid, both of these substances show a tendency to combine with acids and thus retain, strongly, acid dyes.

2. In solutions of such alkalinity as to be on the alkaline side of both isoelectric points, both of the components of the conjugated protein act as acids combining with bases, and in this range basic dyes are strongly retained.

3. At values of pH between these two isoelectric points the protein is acting as a base and the phosphatid as an acid and it is only through this range that the conjugated protein, as an individual, has any stability. Throughout this range the protein would tend to combine with the phosphatid and thus neither would be completely free to combine with either basic or acid dye appreciably. This range we have termed the "isoelectric range" in the preceding paper.

The experimental results are seen to be in striking accord with this very simple picture. The intermediate pH range will vary from organism to organism depending on the specific protein and phosphatid. For example a conjugate protein consisting of lecithin and such a simple protein as serum albumen whose isoelectric point is itself at a pH of 4.7 would be a substance with a comparatively narrow isoelectric range, and would, through the ordinary pH range, tend to retain basic dye. If for the albumen we would substitute a protein such as gliadin, whose isoelectric point is at a pH of about 9.5, we would then have a substance with a much wider isoelectric range and at ordinary pH values there would be little tendency to retain basic dye. Other fac-

tors remaining constant, the first substance cited would in all probability be found to be Gram-positive and the other Gram-negative. Naturally there will be all sorts of intermediate cases with proteins less alkaline and phosphatids less acid.

Throughout this range of loose combination there may be slight residual retention of color since, with both acid dye and phosphatid available with which to combine, the protein compound will depend on the mass relations of the two as well as their relative acidic strengths. In the same way basic dye would compete with protein to combine with the phosphatid. Thus, while there are fairly distinct limits of pH range at which the intensity of retained color suddenly drops off, some residual staining may be expected inside these limits especially with acid dye at lower pH values and basic dye at higher ones. This retention is enhanced by increasing the concentration of the dye solution, giving the mass effect of the dye more nearly an equal chance with the protein or the phosphatid. In our experiments this residual staining was noted, though there was also a distinct range —quite narrow—through which practically complete decolorization seemed to take place in most cases.

Function of mordant

In the paper just preceding it has been shown that any mild oxidizing agent can be used as a satisfactory mordant providing its own pH is not such as to vitiate results. Most of these tend to precipitate the dye, but not all, and there seems very good reason for believing that the real function of the mordant lies in its action on the bacterial matter. The data presented in this preceding paper show pretty distinctly that the general behavior of Gram-positive and Gram-negative organisms is the same, the difference lying for the most part in the limits of the isoelectric range. Anything which will change this range in one type of bacteria and not in another will tend to amplify any original distinction which may be only very slight. From a chemical point of view Jobling and Peterson (1914) have shown that the "lipoid extracted from Gram-positive organisms differs from that extracted from Gram-negative organisms in that the former

contains a much larger proportion of unsaturated acids which have a great affinity for iodine." These would also have an affinity for any other oxidizing agent. This, then, seems to be the point of attack for the mordant. By this mild oxidation the acid character of the phosphatid is intensified just as oxidation tends in general to increase acid properties where it is not vigorous enough to completely destroy.

Table 2 shows the effect of mild oxidation on the ionization constants of a few typical saturated and unsaturated acids. In each case the acids are paired, the oxidation product of any acid

TABLE 2

SATURATED ACIDS		UNSATURATED ACIDS	
Acid	$K \times 10^5$	Acid	$K \times 10^5$
Acetic	1.8	Crotonic	2.0
Hydroxy-acetic	15.0	Alpha-hydroxy-butyric	12.0
Propionic	1.3	Fumaric	93.0
Lactic	13.8	Hydroxy-fumaric	276.0
Butyric	1.5	Maleic	117.0
Alpha-hydroxy-butyric	12.0	Hydroxy-maleic	250.0
Beta-hydroxy-butyric	3.1		
Isobutyric	1.4		
Hydroxy-isobutyric	10.0		

Where the oxidation substitutes or introduces a halogen the effect is even more marked.

being given just below the acid itself. The increase in acid properties by oxidation is quite general. For example, alcohols, which have practically no acid properties, are oxidized to aldehydes which are distinctly though weakly acidic, and these in turn can be oxidized to the organic acids.

The action of the mordant in ordinary differential bacterial staining is selective in that it acts selectively on those cells with a high concentration of unsaturated material. If a more vigorous oxidizing agent were used some of the organisms now classified as Gram-negative would be then classified as positive as was brought out in table 3 of the preceding paper.

It should be noted in passing that Eisenberg (1912) states that it has been found that oxidation destroys the Gram-positive character. In the same paper he reports the use of 5 per cent chromic acid on his bacteria to oxidize aniline to aniline black in contact with the organisms and reports excellent results.

The partially oxidized phosphatid of the Gram-positive organism is thus rendered slightly more acid in character and its affinity for basic dye increased as well as its differentiation from Gram-negative organisms made more marked.

IV. DISCUSSION

The above theory explains many of the anomalies often met with. Sheppe and Constable (1923) found that addition of hydriodic acid to a pH of about 3 rendered Gram-positive organisms amphophile, and more acid made them Gram-negative. Winslow, Falk and Caulfield (1923) find that at this pH the Gram-positive organism *B. cereus* changes cataphoretic migration direction. Burke (1922) found that lactic acid rendered Gram-positive organisms Gram-negative, and both he and Sheppe and Constable agree that the addition of alkali restores the normal reaction.

For many organisms which are not strongly Gram positive, as for example *B. diphtheriae*, the age of the culture is important. These cases of change of Gram character with age are found to be true of organisms which are normally only weakly Gram-positive and are cultured in fermenting media which become acid in reaction as growth proceeds.

Media which permit synthesis of lipoid material seem to have a tendency toward strengthening Gram-positive characteristics. This is in accord with our idea of mordanting. It is much more difficult to bring about the change from Gram-negative to Gram-positive by regulation of the pH than the reverse change, due to the fact that it is more difficult to work with solutions which range from pH 10.0 to 13.0 than the corresponding ones on the acid side, i.e., 1.0 to 4.0.

Robbins (1923; 1924) has shown that in the case of potato tuber tissue as well as the mycelium of *Rhizopus nigricans* and

Fusarium lycopersici their behavior toward dyes, growth rate, etc., could be explained by assuming an amphoteric character with isoelectric points at pH values of 6.0, 5.0, and 5.5 respectively. After discussing the status of the question he states that "the evidence at hand indicates that the conception of the living cell as an amphoteric colloid is worthy of serious consideration."

A final paper in this series will present a brief consideration of bacteriostasis from the point of view of our theory showing how it may be used to explain and correlate many seemingly unrelated phenomena.

In conclusion the authors wish to gratefully acknowledge the valued criticisms and active interest of Dr. M. P. Ravenel.

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THE CHEMICAL MECHANISM OF BACTERIAL BEHAVIOR

III. THE PROBLEM OF BACTERIOSTASIS[1]

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I

The behavior of bacteria toward dyes, so far as staining reaction is concerned, is well accounted for by a modification of Loeb's theory of proteins applied to bacteria by the present authors. (See previous papers in this series.) This same theory, when applied to the known facts of bacteriostasis, will harmonize and render them easily understandable. The simplest method of approach to the problem is to state what would be predicted on the idea that the bacterial cell behaves as a conjugate protein and compare these predictions with known facts.

II. GENERAL

Staining reactions of all bacteria show that they behave as ampholytes tending to retain basic dyes in alkaline solution and acid dyes in acid solution. It might well be expected that the formation of a loose compound between any constituents of the media which are not easily decomposed by hydrolytic action, and thus "digested" by the bacteria, should interfere with the life cycles of such organisms. Bacteriostats, we believe, act in this way. In the following discussion we shall have to treat of hydrolysis, and must therefore differentiate between hydrolytic breaking down or decomposition of a bacteriostat itself, and the hydrolysis of such a thing as a dye-protein compound resulting in the liberation of the original dye from the protein combination.

[1] Contribution from the Division of Physical Chemistry and the Public Health Laboratory, University of Missouri.

III. GRADATIONS IN RESISTANCE TO BACTERIOSTATS

On the basis of staining characteristics bacteria are divided into two classes, Gram-positive which retain basic dye, and Gram-negative which do not. The authors have shown that such a classification, though it is clinically important and will give reproducable results when certain factors are controlled, is arbitrary. In reality there does not seem to be a distinct group of bacteria whose members closely resemble one another in all characteristics but differ distinctly from members of a second group. Rather is there a gradation from one extreme to another, and, while there are organisms which, under practically any set of clinical conditions, will be Gram-positive, and others which will be always Gram-negative, yet the dividing line of such a classification is arbitrary and depends somewhat on the technique used. There will be certain organisms which lie between the two extremes.

Such gradations in character should be brought out more sharply in experiments on bacteriostasis than in staining experiments, since in the former we get a quantitative effect of the dye while in the latter we do not.

Table 1, from data by Kligler, (1918)[2] brings this out in a striking manner. The numbers represent the relative order of sensitiveness to the dye in question. The organism inhibited by the greatest dilution, *B. subtilis*, is taken as the standard in every case and numbered 1. Where equal sensitiveness was found for different organisms the same number is used for them.

The numbers represent ratios of inhibiting concentrations of dye based on the concentration required by the most sensitive organism. For example the number 20 means that the organism to which it applies required twenty times as high a concentration of dye for inhibition as did *B. subtilis*, the arbitrary standard.

[2] The data presented in this paper by Kligler seems about the most reliable *comparative* data available. Kligler recognizes and specifically describes his control of all of the following factors: (1) composition of medium; (2) reaction of medium; (3) adaptation of the organism to the test fluid; (4) approximate number of organisms inoculated. For this reason this data seems best adapted to use for conclusions as to comparative effects, and we have made much use of it.

The table includes values for all the triphenylmethane dyes used by Kligler.

It will be noted that, with the exceptions of *Bact. coli* and *B. proteus* which seem very similar in sensitiveness, the order of sensitivity is always the same.

The significant fact brought out by the table, however, is that there is *no* distinct jump between Gram-positive and Gram-negative organisms. Indeed there seems to be in many cases a greater difference in sensitiveness between pairs of Gram-negative

TABLE 1

DYE	GRAM-POSITIVE		GRAM-NEGATIVE				
	B. subtilis	*S. aureus*	*B. dysenteriae* S.	*B. proteus*	*Bact. coli* B	*Bact. typhosum*	*Bact. aerogenes*
Brilliant green	1	3.75	7.5	22.2	27.3	29.4	150.0
Crystal violet	1	4.0	10.0	20.0	40.0	47.0	>67.0
Methyl violet B	1	5.0	22.8	47.0	53.3	66.6	>67.0
Victoria green	1	5.0	7.1	125.0	71.3	125.0	>125.0
Malachite green	1	4.0	8.0	114.0	89.0	113.0	>200.0
Dahlia	1	2.5	18.2	36.4	30.8	>45.0	>45.0
Fuchsin	1	1.67	4.5	41.6	41.6	41.6	>63.0
Methyl green	1	1.67	6.2	18.5	41.6	41.6	41.6

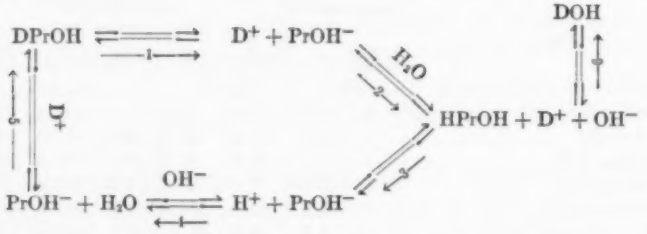
organisms than between the groups. All the dyes used were basic. It is thus misleading to state, as is done by so many authors. (Krumwiede and Pratt, 1914; Smith, 1922; Churchman, 1923, Davis, 1921), that Gram-positive organisms are selectively inhibited by basic dyes. For example Kligler finds that 1:2,000,000 brilliant green will inhibit *Bact. dysenteriae* S, 1:200,000 crystal violet will inhibit *B. proteus*, etc.

IV. ACTION OF BASIC SUBSTANCES

If the bacteriostatic action of basic dyes is of the nature of a loose combination of bacterial matter with difficultly "digestible" dye material, we should expect that such bacteriostatic action need not be confined to basic dyes, but that other basic substances of similar nature should have a similar effect.

This is shown by Kligler (1918). He lists eleven basic substances, other than dyes, which inhibit even Gram negative bacteria (those listed in table 1) at dilutions greater than 1:1000. There are also listed others less effective. The effective ones include such substances as substituted anilines and toluidines, quinoline, quinaldine, alphanaphthylamine, etc. These substances are more effective even than some of the basic dyes themselves, as for example auramine, methyl green and aniline blue.

A fair picture of some of the important equilibria in a solution containing dye and protein may be presented in the following scheme:



Here the amphoteric protein is represented by the type formula HPrOH, the basic dye by DOH, the dye-protein compound by DPrOH and the ionization products of this compound by D^+ and and $PrOH^-$.

Maximum bacteriostatic effect will be produced by maximum formation of the dye-protein compound, DPrOH. This, however, tends to ionize (process 1), and, since the free protein acid, at least, is very weak, hydrolysis will tend to take place (process 2). In general the strongly bacteriostatic dyes are ionized to a much higher degree than the free protein so we have represented them as ionized. The unionized dye base will, however, tend to form to a certain extent (process 6). Even though the protein is only slightly ionized, nevertheless it will be in equilibrium with its ionization products as an acid (process 3), and an increase in alkalinity will aid the formation of the acid protein ion (process 4). Finally, of course, this protein ion, in the presence of dye ion will tend to form the dye-protein compound (process 5).

From a study of these equilibria one might predict the nature of the influence of at least three factors.

1. An increase in pH, yielding a higher concentration of hydroxyl ion, will tend to inhibit process 2 and would at the same time favor process 4, in both cases aiding the bacteriostatic effect of the basic dye. Such a change in pH will also aid process 6 thus tending to decrease the bacteriostatic effect. This last named tendency will be more than offset, however, by the effect on process 4, since the protein will be much weaker as an acid than the dye is as a base.

Experimentally, in the comparatively few instances where the influence of hydrogen ion concentration has been studied, the

TABLE 2

Aniline	300	450	Aniline	300	450
Methyl aniline	650	950	Ethyl aniline	1,000	1,350
Dimethyl aniline	1,350	1,750	Diethyl aniline	4,500	7,000
o-toluidine	475	600	p-toluidine	475	600
Methyl o-toluidine	1,100	1,400	Methyl p-toluidine	1,100	1,400
Dimethyl o-toluidine	1,550	2,100	Dimethyl p-toluidine	2,500	3,500
Quinoline	1,400	1,600			
Tetrahydro-quinoline	1,600	2,100			
Quinaldine	2,600	3,400			

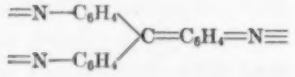
inhibiting power of basic dyes has in all cases been found to be increased by increase in alkalinity and decreased by increase in acidity (Davis, 1921; Graham-Smith, 1919; Beckwith, 1921; Davis and Harrell, 1918; Browning, Gulbransen and Kenneway, 1920; Fleischer and Amster, 1923). Even in the case of basic bacteriostats other than dyes Dernby and Davide (1923) found that the "bactericidal action," as it is termed, of the quinine alkaloids increases with increasing alkalinity, while Kligler (1918) finds the same to be true for caffeine.

2. The basic strength of the dye should affect its bacteriostatic power, other factors being equal. The stronger the dye is as a base the smaller will be the tendency of both processes 2 and 6 to take place, and thus, for any closely related series of dyes or

other basic substances, the bacteriostatic power should increase with the basic strength. The substitution of alkyl groups for hydrogen atoms in ammonium hydroxide is known to increase the strength of the latter as a base. Ethyl groups are more effective than methyl and propyl groups are more effective than ethyl, etc. Table 2, from Kligler's data (1918) is quite suggestive. The numbers represent the inhibiting dilutions of the designated base for *Bact. aerogenes* and *Bact. dysenteriae* S respectively.

The relation between ionization and toxicity of strong acids and bases was rather firmly established by Paul and Krönig (1896) (1897). For organic acids and bases other factors seem to enter, however, and different groups of compounds will differ markedly in effect. Some of the factors controlling toxicity are as yet unrecognized, just as we fail to recognize the reason for the strong toxicity of the barium ion physiologically in spite of the fact that the closely related calcium and magnesium ions are not toxic. We fail to account for the fact that arsenic salts are so much more toxic than phosphates, that oxalic acid is toxic and acetic acid is not, that the carbon-nitrogen bond in the form of cyanide is toxic while in amino-acids it is comparatively non-toxic, etc. Inside of any one group, however, the same rule seems to hold, i.e., toxicity parallels electrolytic strength. One of the most common groups met with is that of the triphenylmethane dyes.

Fairbrother and Renshaw (1922) suggest that the antiseptic property of these substances is a function of the complex



This seems approximately true except that it is not necessary to have three amino groups present. The important part of such a complex seems to be the double-bonded pentavalent "onium" nitrogen. While it cannot be said that substituents at other points on the molecule do not affect results, nevertheless there is reason to believe that the controlling factor in determining the bacteriostatic power of a triphenylmethane dye for any one

TABLE 3

DYE	FORMULA	LIMITING DILUTION FOR	
		B. subtilis	*S. aureus*
Brilliant green	$(C_2H_5)_2N-C_6H_4 \diagdown \diagup C_6H_5 \diagdown C=C_6H_4=N(C_2H_5)_2Cl$	15,000,000	4,000,000
Malachite green	$(CH_3)_2N-C_6H_4 \diagdown \diagup C_6H_5 \diagdown C=C_6H_4=N(CH_3)_2Cl$	4,000,000	1,000,000
Victoria green	$(CH_3)_2N-C_6H_4 \diagdown \diagup Cl_2C_6H_3 \diagdown C=C_6H_4=N(CH_3)_2Cl$	5,000,000	1,000,000
Methyl-violet B	$(CH_3)_2NC_6H_4 \diagdown \diagup CH_3HNC_6H_4 \diagdown C=C_6H_4=N(CH_3)_2Cl$	4,000,000	800,000
Crystal violet	$(CH_3)_2NC_6H_4 \diagdown \diagup (CH_3)_2NC_6H_4 \diagdown C=C_6H_4=N(CH_3)_2Cl$	4,000,000	1,000,000
Dahlia	$C_2H_5HNC_6H_4 \diagdown \diagup C_2H_5HNCH_2C_6H_3 \diagdown C=C_6H_4=NHC_2H_5Cl$	2,000,000	800,000
Fuchsin	$H_2NC_6H_4 \diagdown \diagup H_2NCH_2C_6H_3 \diagdown C=C_6H_4=NH_2Cl$	500,000	300,000

organism at any one pH is the nature of the groups attached to this "onium" nitrogen.

The most carefully controlled experiments on a comparatively large number of dyes of this group are those of Kligler (1918) who studied the effect of the dyes in a medium at a pH of 7.1. Table 3 gives his data for the only two Gram-positive organisms he studied.

Section VII of this paper will discuss the reason for the use of Gram positive organisms only in this comparison.

The brilliant green, with two ethyl groups on the "onium" nitrogen, should be most strongly basic. It is by far the most effective on both the above organisms. The extremely powerful effect of this dye has been also noted by Norton and Davis (1923), Browning and Gulbransen (1919), Levine (1921), Salter (1919), Conradi (1908), and others. The next four dyes all have two methyl groups on the "onium" nitrogen, though the remainder of the molecule varies a good deal. They are seen to be inhibitive to about the same degree in spite of the fact that some have amino groups while others have only hydrogen atoms in the same place and still others have even chlorine atoms on certain of the benzene rings. Dahlia has one ethyl group in place of two methyls and might be equally effective or even slightly more so. Actually it seems slightly less so. Fuchsin, with only hydrogen atoms is still less powerful. Data for Gram-negative organisms only in the case of aniline blue, a dye which has a slightly acidic phenyl group on the "onium" nitrogen indicates that it is still less powerful than fuchsin, while methyl green, which has one of the amino nitrogens in the form of a quaternary ammonium salt has also lost its power.

In any study of the relation between toxicity and chemical constitution, the position as well as the nature of the substituent groups is very important. In the case of the above dyes the position where substituents most easily influence bacteriostatic power is on the "onium" nitrogen (Crossley, 1919). This explains the confusion in the literature regarding the effect of adding ethyl or methyl groups. Such confusion is enhanced by failure to expect the reverse effect of such substituents on acid dyes. In

general it may be stated that positive groups tend to increase the inhibitive power of basic dyes when they are so substituted as to increase the basic strength of the dye. Otherwise they may have little effect. Negative groups, such as phenyl, nitro, nitroso, carboxyl, halogens, etc., have the reverse action. (Fairbrother and Renshaw, 1922; Simon and Wood, 1914a and 1914b). Norton and Davis (1923) find that adding two methyl groups to thionin, producing toluidin blue, increases the bacteriostatic power by ten times. Such effect, it must be remembered, will be true for basic dyes only.

3. Finally, from our set of equilibria, we see that our protein should be easily liberated from its combination if we can remove, in any way, the dye ion or free dye base. This indicates that, unless some other agent is active in actually destroying bacterial protein during the period when its growth is inhibited by the bacteriostatic action of the dye, the organism is merely awaiting opportunity to develop. A striking example of such a case can be shown by taking the mercuric ion in place of basic dye. Engelhardt (1922) finds that staphylococci which have been treated for seventy-two hours with one per cent solutions of $HgCl_2$ can be rendered capable of reproduction by treatment with sulphides. Charcoal or sulphides will produce the same effect on anthrax spores treated at 37° for thirty-five days with 0.1 per cent $HgCl_2$ or eleven to seventeen days with 5 per cent $HgCl_2$. In either case the removal of the mercury ion aids processes 1 and 2 of our diagram, liberating free bacterial protein. Churchman (1921) found that *Bacillus anthracis*, stained with gentian violet and injected "in enormous doses into a mouse, revive after twenty-four days of complete quiescence and kill the animal, the controls having died in thirty-six hours."

V. ACTION OF ACID SUBSTANCES

There is a general feeling that, in the words of Simon and Wood (1914a) "acid dyes, irrespective of their chemical structure and color, do not possess this inhibitory power." The same conclusion is voiced by Norton and Davis (1923), Davis (1921), and in modified form by Fairbrother and Renshaw (1922).

If inhibitive action is chemical, as we believe, and if proteins are amphoteric, then there is no reason why acid dyes should not have bacteriostatic effect. They will not, however, exhibit their maximum effect under the same conditions as basic dyes. Under the latter conditions a very much higher concentration of acid dye would be required.

From a theoretical point of view, however, one might construct a diagrammatic set of equilibria for acid substances, analogous in all respects to that which is given for basic substances, and one could at once see from such a diagram that for acid dyes to be effective an increase in acidity would be necessary. That there is such an inhibiting effect on the part of acid dyes is recognized by Churchman (1922; 1923a) in his use of acid fuchsin. He attributes its action to specific chemical effects of sulphonic acid groups, but other acid substances not containing these groups, e.g., quinone and hydroquinone, have been shown to have more powerful action than many acid dyes (Graham-Smith, 1919).

The effect of pH on the power of acid bacteriostats has also been fairly well established by the comparatively few who have studied the relationship. Graham-Smith (1919) found that quinone was more effective in acid than in neutral or alkaline solution. Acid fuchsin (Beckwith, 1921), and the acid chlormercury fluorescein (Davis, White and Rosen, 1918) are more active in acid solution. Fleischer and Amster (1923) come to the general conclusion that "the action of the acid dyes is increased by further acidity and that of the basic dyes by a decrease in acidity."

Furthermore the effect of substituent groups on acid bacteriostats is the reverse of that on basic ones. Alkyl groups, such as methyl, ethyl, etc., tend to decrease their power (Fairbrother and Renshaw, 1922) if they produce any noticeable effect at all (Hirschfelder and Pankow, 1921). The widespread exclusive use of basic dyes has not so far stimulated much careful comparative study of acid dyes at lower values of pH, and the comparative effect of substituting various negative groups on acid dyes is not well known, as is their effect on basic dyes. The necessity of carrying out such a study at pH values lower than those of ordi-

nary media, if such a study is to be valuable, will be discussed later.

VI. OTHER CHEMICAL FACTORS

On the basis of specific selective action by bacteriostats the factors considered may be, and usually are, limited for any one dye or bacteriostat to (*a*) type of organism, and (*b*) greatest inhibiting dilution.

If the phenomenon is of a general chemical nature others must be considered. The most important of these are (1) composition of the medium, (2) mass effect of bacteria, and (3) reaction of the medium. Other possible factors, such as the color of the dye and the penetrability have been shown not to effect inhibitive power greatly, nor would these factors be expected to be important on a chemical basis. Color in itself will not determine the extent of dye-protein combination. Also it is obviously possible for a dye solution to penetrate without combination. Indeed, for certain of the enormously high dilutions of dyes sometimes found effective, it might well be expected that combination at the surface would tend to use up the dye as the liquid penetrated into the organism. Therefore observations such as that of Churchman (1923) that "static effects may be produced without penetration . . . . but it is equally true that penetration may occur without stasis," lends support to the chemical theory.

Of the other factors listed above number 3, the effect of the reaction of the medium, has been examined and found to be in entire accord with chemical theory. The data on the other two factors point just as strongly in the same direction in the one case and are in harmony with such a view in the other.

1. Composition of media

In the case of this factor two distinct effects are to be looked for. In slightly buffered media, where slight growth of the organism is accompanied by a change in pH, either lowering this in fermenting media, or tending to increase it in media containing exclusively nitrogenous compounds such as peptone, the primary

effect on bacteriostasis may be due to changes in pH which have been discussed.

If, however, the action of the bacteriostat is chemical, and if conditions are such that basic antiseptics are effective, then basic constituents of media should aid the basic dye, and in their presence the inhibiting dilution should be greater. Amphoteric constituents might have either the same or the opposite effect depending on which side of their isoelectric point the media happened to be, while acid constituents, by tending to combine with the basic dye, should tend to neutralize its effect on the bacteria. The reverse would, of course, hold for antiseptics which are acidic.

While the effect of the composition of the media has been vaguely recognized and some work has been done along this line, it has not been planned with the above theory in mind, and the obvious complexity of this factor renders the interpretation, from the point of view of our theory, of the results which have been published less easy, and their weight as confirmatory evidence less clear cut. The results of workers in this field are, however, in complete harmony with a chemical theory. Kligler (1918) states that the higher the concentration of organic nitrogenous compounds (peptone) in the medium, the lower is the effective concentration of the dye. He worked with basic dyes only. While peptones are still amphoteric, they seem a good deal more basic than the proteins from which they come (Mathews, 1916) and results with peptone media are less equivocal than those with media employing various sera or meat extractives. Among the few experiments which permit direct interpretation are those of Graham-Smith (1919). He found that typical basic dyes such as crystal violet were more effective in bacteriostatic action when used in neutral meat extract than when used in neutral agar. While the nature of the meat extract may be questionable the nature of agar as a carbohydrate is unquestionable and its nature will be more nearly acidic. With the acidic substances quinone and hydroquinone the opposite is true. They were more effective when used with the agar. He also showed that the presence of gelatin or egg albumin, whose isoelectric points are at a pH of

4.7 and thus whose properties would be acidic in neutral solution, tended to weaken the effect of basic dye but did not weaken the effect of quinone.

Very striking in this connection are the results of Winslow and Dolloff (1922) on the effect of bile salts on the toxicity of rosolic acid, gentian violet and brilliant green. These bile salts are sodium compounds of bile acids, as for example cholic acid. They should, therefore, be expected to hinder the action of basic dyes and aid, if they affect at all, that of acid dyes. These authors find that the inhibitive powerful of rosolic acid is unaffected, that the power of gentian violet is reduced from five to fifty times, depending on the organism, by the bile salts, and that the power of brilliant green is reduced by these salts from two hundred to two thousand times. This is in striking quantitative agreement with the data in table 3, showing brilliant green, from its structure, to be the strongest base, and thus to have the greatest tendency to combine with acid such as cholic acid.

Finally the finding of Wels (1922) is suggestive when he states that "the addition of small pieces of tissue decreases the bactericidal power of serum-dye solutions."

It is the authors' opinion that this factor furnishes the explanation for the well known fact that applications of "in vitro" experiments to "in vivo" conditions many times fail to yield the expected results. As stated by Browning and Gulbransen (1919), "Methods of testing antiseptics in vitro . . . . . may fail altogether to afford a true estimate of the potency of a substance for therapeutic purposes."

2. Mass action of bacteria

In the case of this factor the evidence is a good deal more clear cut and directly applicable. If bacteriostatic action is chemical the actual quantity of bacterial matter should affect the limiting inhibitive dilution. Qualitatively, Lange (1922) states that "disinfectants tested with large numbers of bacteria or thick emulsions are inhibited in their action." This he attributes to adsorption processes. Table 4 gives data by Gay and Beckwith

(1922) on *Streptococcus hemolyticus* using acriflavine diluted in broth, and also data by Graham-Smith (1919) on *Staphylococcus aureus* using homoflavine and quinone in a meat extract medium.

These numbers are merely comparative, and, while each set of experiments was run under uniform conditions, the two sets are not to be compared. As would be expected on the basis of a chemical theory, here is one case in which the variation of an acid antiseptic like quinone is the same, and not the reverse of, the variation of basic substances.

Results similar to those above have been obtained by Gay and Beckwith (1922) for various doses of typhoid bacillus and new fast green, also by Browning and Gulbransen (1919) for both *Staphylococcus aureus* and *Bact. coli* with both acriflavine and proflavine.

TABLE 4

DILUTION OF COCCI	ACRIFLAVINE	HOMOFLAVINE	QUINONE
1.0	8,000		20,000
0.1	64,000	100,000	50,000
0.01	128,000		
0.001	256,000	250,000	

Churchman (1920) finds that a thick suspension of Gram-negative *Bact. coli* will grow equally well on plain agar and on gentian violet agar. But if a weak dilution of the suspension be used, a few colonies will appear on the plain agar but none at all on the gentian violet agar. Explanation of Churchman's "communal activity" of bacteria (1920) may possibly be found in this mass factor. Inasmuch as dead bacterial bodies need not differ appreciably from living organisms in chemical composition, this mass effect offers also a good explanation of Churchman's observation that dead bacterial bodies, interposed between living organisms and gentian violet media, partially negative the effect of the dye on Gram-positive organisms (these are the ones on which its inhibiting action is most marked) and allow them to grow.

Finally, if any chemical explanation holds, one should be able to show that even in the rather startling dilutions of dye found

effective, the stoichiometrical amount of dye is sufficient for the bacterial protein. Only one piece of work permits such calculations. Graham-Smith (1919) determined the number of organisms in the amount of culture he employed for inoculation as between 2000 and 4000. Hiss and Zinsser (1916) give the average diameter of the ordinary pus coccus as from 0.8 to 1.2 microns with a maximum of about 2 microns, and the average protein content of many bacteria as from 8.5 to 14 per cent. From Proctor's data on gelatin, Wilson (1917) has calculated its equivaent weight at 768. (Its molecular weight is of course much greater.) Wintgen and Krüger (1921) give the value as 839. One of the authors has calculated from data on the combination of fibrin with acid (Tolman and Stearn, 1918) that its equivalent weight in less than 2000. If for safety's sake we take the limiting cases, we shall have, assuming a radius of 1 micron per organism, a specific gravity of 1,4000 organisms per inoculation, 12 per cent protein in the organism, and an equivalent weight of 800 for the protein, for the number of chemical gram equivalents of protein per inoculation, since 1 micron equals 0.0001 cm.: $4 \times \pi \times (0.0001)^3 \times 1 \times 4000 \times 0.12 \times 1/800$ or approximately 10^{-11} gram equivalents.

The greatest inhibiting dilution for such an inoculation was 1:10,000,000 crystal violet in 5 cc. of broth. The molecular weight of this dye is about 400 (407), thus the number of equivalents of dye present, assuming a specific gravity of 1 for the broth, was: $5 \times 10^{-7} \times 1/400$ or approximately 10^{-9} gram equivalents. The ratio, $10^{-9}/10^{-11}$, or 10^2 indicates that even in this case there is actually present one hundred equivalents of dye to one of bacterial protein.

VII. VARIATIONS IN BACTERIOSTATIC ACTION WITH THE TYPE OF BACTERIA

It was pointed out in section III that there are gradations of dye sensitiveness from very striking cases among strongly Gram-positive organisms to other species which are very resistant to the action of dyes. These latter are almost always Gram-negative.

This has caused general statements as to selective effect of dyes on Gram-positive organisms.

The general behavior of these two great classes of organisms toward the bacteriostatic action of dyes, if the phenomenon is chemical in nature, may be expected to be controlled by the same factors which determine the retentive power of these groups toward dyes.

The authors have presented data which indicate that the fundamental difference, chemically, between strongly Gram-

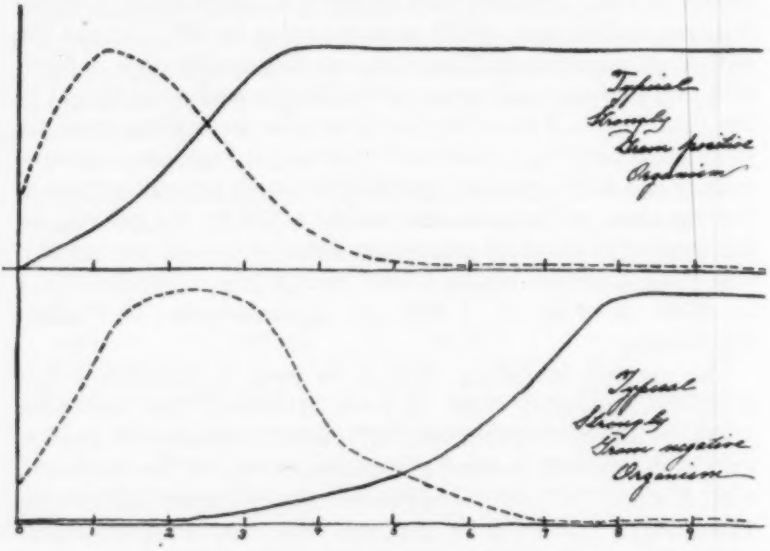


FIG. 1

positive organisms and strongly Gram-negative ones lies in the difference in pH limits of an isoelectric range characteristic of each organism.

Figure 1 is a diagrammatic sketch of this difference. Abscissae are pH values from 0 to about 10, ordinates are functions of the tenacity with which basic dyes (unbroken line) and acid dyes (broken line) are held. For organisms whose Gram character is pronounced there is little doubt as to the validity of the gener-

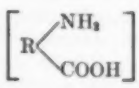
alization that basic dyes, in media of ordinary pH values, will be much more effective on Gram-positive organisms than on Gram-negative. For organisms whose Gram-character is less pronounced there may well be exceptions to such a rule due to the fact that, in bacteriostatic action, it is the unmodified bacterial protein with which the dye must combine, while in the Gram stain a mordant is used which has been shown by us to have the effect of shifting the isoelectric range to a lower value of pH.

With acid dyes, in media of ordinary reaction, figure 1 indicates that no general prediction is possible regarding group specificity. Certainly acid dyes, at ordinary values of pH, will as a class be very much less effective than basic dyes, which is, of course, the case; but as to which group is more susceptible there is difference of opinion. This our work indicates may well be. Churchman (1922; 1923) seems to think that Gram-negative organisms are more sensitive to acid fuchsin than Gram-positive spore-bearing aerobic organisms. On the other hand, Smith (1922) states that he has tested many species of bacteria with acid fuchsin as well as erythrosin, eosin and other acid dyes, and the Gram-negative have invariably shown greater resistance than the Gram-positive.

VIII. THE CHEMICAL MECHANISM OF BACTERIOSTATIC ACTION

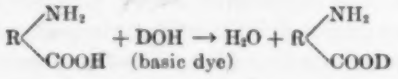
Simon and Wood (1914b) have come the nearest to what we believe to be the chemistry of bacteriostasis. For the action of basic dyes they say, "The most plausible inference would be to assume the existence of corresponding acid groups in the structure of the organism with which basic groups would tend to unite."

The amino-acid composition of proteins and allied amphoteric substances furnishes just such acid groups. If we think of the protein molecule as having the property of an amino-acid, and write a type formula, as Loeb does (1922)



we have, in the language of Simon and Wood, receptors for either acidic or basic substances.

On the alkaline side of the isoelectric point the reaction with the basic dye would be represented thus:

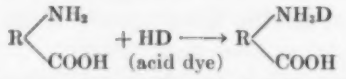


The dye molecule is only very difficultly destroyed so that the organism finds itself unable to liberate that portion of the dye which it might utilize in its own metabolism, and by this liberation free again this "receptor" or point of attack for basic nitrogenous nutrient material. This would bring about starvation of the organism, not necessarily to the extent of actually killing it at once, but in the sense of inhibiting growth and multiplication.

On the acid side of the isoelectric point it might be expected that acid dyes should be just as effective as are basic dyes on the alkaline side. For a simple protein they might be, but the authors have pointed out that staining characteristics of bacteria indicate an isoelectric range between the isoelectric points of bacterial protein and bacterial lipoid, through which the protein would act as a base and the lipoid as an acid.

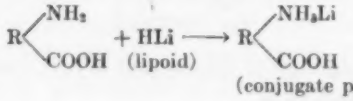
Basic dyes find their task very simple, for a solution on the alkaline side of the isoelectric point of bacterial protein is also on the same side of the isoelectric point of the much more strongly acidic lipoid, and there is thus no interference with action. Throughout the isoelectric range, however, the lipoid is working against an acid dye and even though the reaction is favorable for dye-protein combination it is also favorable for lipoid-protein combination into a conjugate protein, and we have both lipoid and acid competing for the protein. These two reactions which tend to take place simultaneously may be represented:

(1)



[Analogous to the reaction $NH_3 + HCl \rightarrow NH_4Cl$]

(2)



(conjugate protein)

and a very much greater quantity of acid dye would have to be used for the same inhibitive effect than of basic dye under the former set of conditions.

For acid dyes to have an effect commensurate with the well known effectiveness of basic dyes when used properly, it would be necessary to lower the pH to a value below the isoelectric point not only of the protein but also of the lipoid. In general this will throw the reaction over to an acidity so great that inhibition of growth will take place without the addition of dye at all, so that the practical application of high dilutions of acid dyes seems limited.

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HEATING TO FACILITATE MEASUREMENT OF PRECIPITATES

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Differences in growth can not be accurately measured by turbidity standards when the organisms produce copious or tough films or either viscous or coarse flaky sediment. Even with violent shaking the particles obtained may be too large or too uneven in size to assure accurate turbidity readings by ordinary transmitted light or with the nephelometer. In some cases, the film or pellicle particles are so light or so buoyed up by bubbles adhering to their fragments that they can not be made to distribute themselves equally throughout the liquid.

Important investigations, e.g., on the stimulating and also the inhibitory effect of chemicals on bacterial growth have been handicapped by this difficulty, as it has been found impossible to include certain organisms in such comparative studies. Thus, workers have often, therefore, been forced to omit just the organisms which would increase the range or variability of the phenomena under investigation.

As reported earlier,[1] such difficulties may be reduced to a minimum by heating the tubes before reading for five to ten minutes in a water bath at 100°C. The comminution of the coarser or tougher particles is greatly hastened by frequent hard shaking of the tube, e.g., hitting the butt vigorously against the palm of the hand, as is commonly done in mixing dilutions, etc.

A second mention of this process seems justified because it has unexpectedly proven valuable in another field—in securing complete and compact sedimentation of yeasts, now so widely used in vitamine testing.

[1] Broadhurst, New York Chapter, Soc. Amer. Bact., December 19, 1923.

Comparisons of yeast growth (and of vitamine efficiency) are often made by centrifuging the yeast cultures, and estimating the difference in the precipitates when collected in the graduated bases of the centrifuge tubes. With some varieties of yeasts the flaky sediment and the unusually adhesive fibrous pellicle resist even prolonged shaking or centrifuging and accurate comparative determinations are not possible from the precipitates which are obtained. This is true of at least two budding yeasts which are very responsive to vitamine additions, but which form a pellicle which sometimes adheres tenaciously to the tube for a height of at least $\frac{1}{2}$ inch above the culture medium. Heating, however, as described in the next paragraph, removes these difficulties.

The tubes are immersed in boiling water for ten to fifteen minutes. At the end of five or six minutes, the tubes are taken out singly and shaken or knocked vigorously against the palm of the hand an equal number of times. Each tube is promptly replaced in the water bath and shaken again at the end of the heating period, taking care to wash any particles on the side of the tube down into the liquid with the last shaking of the tube.

Then (or at any later convenient time, if preceded by thorough shaking) the contents of each tube are poured into a graduated sedimentation or centrifuge tube, following which complete and compact sedimentation may be secured by gravity only or by centrifuging. Either method yields a perfectly clear supernatant fluid.

Since each set of vitamine tests necessarily carries with it one or more controls, there can be no valid objection to heating the yeast cultures in this manner. While the larger particles seem to be dissolved there is apparently only a decrease in the size of the ultimate particles and not a loss in total suspended matter, for the differences in amount of growth, as measured by these heated precipitates, tally with expectation (vitamine strength) and sharper differentiation (both within a series and against controls) is obtained by such heating than occurs with contemporary unheated cultures.